Mariana dos Santos Alves **The role of biofilms in *Klebsiella pneumoniae* multi-drug resistance**

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The role of biofilms in *Klebsiella pneumoniae* multi-drug resistance

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Trabalho efetuado sob a orientação da
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e da
Doutora Ana Margarida Sousa

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STATEMENT OF INTEGRITY

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O papel dos biofilmes na multirresistência de *Klebsiella pneumoniae* a antibióticos

Resumo

A *Klebsiella pneumoniae* é uma bactéria Gram-negativa multirresistente (MDR) que ganhou uma enorme importância nesta crise global de resistência a antibióticos em comparação com outros patogénicos MDR devido ao surgimento de β-lactamases de espectro estendido (ESBL) e carbapenemases (CP-KP). A infeção causada por ESBL e CR-KP está associada a um aumento da taxa de mortalidade e morbidade devido à falta de tratamentos eficazes. A formação de biofilme exacerba a tolerância a antibióticos dessas variantes genéticas, conferindo uma resistência multifatorial.

O objetivo deste trabalho consistiu no estudo do papel dos biofilmes em estirpes MDR de *K. pneumoniae* para determinar uma possível associação entre perfis de resistência ou expressão de fatores de virulência com a capacidade de formação biofilmes e disseminação de resistência a outras espécies dentro dos biofilmes.

Neste estudo, 26 estirpes de *K. pneumoniae* com perfis de resistência bastante distintos, incluindo as variantes ESBL e CR-KP, foram usadas para avaliar a expressão dos fatores de virulência, capacidade de formação de biofilme, diversificação clonal e o potencial de disseminação de resistência a antibióticos para *P. aeruginosa.* Entre esses determinantes, verificou-se que as variantes de ESBLs tinham mais propensão a formar biofilmes, o que reforça o papel dos biofilmes na resistência da *K. pneumoniae*. Por esse motivo, a formação de biofilme foi mais profundamente explorada, a fim de obter conhecimento sobre sua fisiologia e seu papel na resistência a antibióticos. Portanto, a composição da matriz de biofilme foi inferida usando dois tratamentos enzimáticos diferentes com DNase I e proteinase K e os resultados revelaram que a matriz apresentava conteúdo proteico significativo, mas DNA extracelular limitado. A composição da matriz não parece estar correlacionada com o perfil de resistência das estirpes de *K. pneumoniae*. Neste estudo, também foi avaliado o potencial dos biofilmes em proporcionar condições favoráveis à disseminação da resistência a outras espécies bacterianas. Após 24 e 72 h de partilha do ambiente do biofilme, a *P. aeruginosa* não pareceu adquirir tolerância adicional a antibióticos, mesmo de estirpes de *K. pneumoniae* produtoras de ESBL. Contudo, a *P. aeruginosa* desfrutou dos mecanismos de resistência de *K. pneumoniae* em biofilmes que persistem após tratamentos agressivos com antibióticos.

Os resultados deste projeto permitiram determinar claramente que estirpes produtoras de ESBL tinham mais capacidade de formar biofilmes, destacando a sua importância. Além disso, os resultados revelaram que a matriz desempenhou um papel importante na resistência a antibióticos, diminuindo em favor da sobrevivência celular. Essa matriz também parecia conferir essa proteção a outras espécies bacterianas que vivem nos biofilmes de *K. pneumoniae*. Os resultados deste trabalho ajudarão a elaborar estratégias antibióticas eficazes para controlar a disseminação de *K. pneumoniae*.

Palavras-chave: formação de biofilme; *Klebsiella pneumoniae*; resistência a antibióticos;

The role of biofilms in *Klebsiella pneumoniae* multi-drug resistance

Abstract

Klebsiella pneumoniae is a multi-drug resistant (MDR) Gram-negative bacterium that has gain importance in the global crisis of antibiotic resistance in comparison with other MDR pathogens due to the emergence of extended-spectrum β-lactamases (ESBL) and carbapenem-resistant *K. pneumoniae* (CR-KP). The infections caused by ESBL and CR-KP are associated with increased mortality and morbidity rate due to the lack of effective treatments. Biofilm formation exacerbates the antibiotic tolerance of these genetic variants conferring a multifactorial resistance.

The objective of this work consisted in the study of the role of biofilms in the *K. pneumoniae* formed by MDR strains to further determine a possible association between resistance profiles or virulence factors expression with ability to form biofilms and disseminate resistance to other species within biofilms.

In this study, 26 *K. pneumoniae* strains with distinct resistance profiles, including ESBL and CR-KP variants, were used to evaluate the virulence factors expression, biofilm formation ability, clonal diversification and the potential to disseminate antibiotic resistance to *P. aeruginosa*. Among these determinants, it was found that ESBLs variants had more propensity to form biofilms, which reinforced the role of biofilms in *K. pneumoniae* resistance. By this reason, biofilm formation was further deeply explored in order to gain insights about its physiology and their role in antibiotic resistance. Therefore, biofilm matrix composition was inferred using two different enzymatic treatments with DNase I and proteinase K and the results revealed that matrix had significant protein content but limited extracellular DNA. The matrix composition did not seem to be correlate with the resistance profile of *K. pneumoniae* strains. In this study, it was also evaluated the potential of biofilms to provide favorable conditions to the dissemination of resistance to other bacterial species. After 24 and 72h of sharing biofilm environment, *P. aeruginosa* did not seem to gain additional antibiotic tolerance from *K. pneumoniae*, even from ESBL-producing strains. Nevertheless, *P. aeruginosa* enjoyed the mechanisms of resistance of *K. pneumoniae* in biofilms persisting after aggressive antibiotic treatments.

The results of this project allowed to clearly determine that ESBL-producing strains had more ability to form biofilms, highlighting the importance of biofilms. Moreover, the results revealed that matrix played a major role in antibiotic resistance being diminished in favor of cell survival. The matrix also seemed to confer this protection to other bacterial species that lives in *K. pneumoniae* biofilms. The results of this work will help to design effective antibiotic strategies to control the spread of *K. pneumoniae*.

Keywords: antibiotic resistance; biofilm formation; *Klebsiella pneumoniae*;

Index

Abbreviations IX

List Of Figures XI

List Of Tables XIII

Chapter 1. Introduction 1

1.1. Scope Of The Thesis 1

1.2. Objective 1

1.3. General Outline 1

Chapter 2. State Of The Art 2

2.1. *Klebsiella pneumoniae* 2

2.2. Antibiotic Resistance 3

2.2.1. Extended Spectrum β-Lactamase-Producing *Klebsiella pneumoniae* 8

2.2.2. Carbapenem-Resistant *Klebsiella pneumoniae* 11

2.2.3. Multiple β-Lactamase-Encoding *Klebsiella pneumoniae* 14

2.3. *Klebsiella pneumoniae* Virulence Factors Expression 15

2.3.1. Capsule 16

2.3.2. Type 1 And 3 Fimbriae 17

2.3.3. Lipopolysaccharides 18

2.3.4. Siderophores 19

2.3.5. Biofilm Formation 20

2.3.6. Clonal Diversity 22

2.3.7. Hypermucoviscosity 23

2.4. Dissemination Of *Klebsiella pneumoniae* Antibiotic Resistance 25

2.5. Management Of *Klebsiella pneumoniae* Infections 26

Chapter 3. Materials And Methods 28

3.1. Bacterial Strains And Cultures Conditions 28

3.2. Antimicrobial Susceptibility Test Of *Klebsiella pneumoniae* Isolates 28

3.3. Phenotypic Detection Of Extended-Spectrum β-Lactamases 30

3.4. Characterization Of The Virulence Factors Expression Of *Klebsiella pneumoniae* Isolates 30

3.4.1. Phenotypic Detection Of Hypermucoviscosity 30

3.4.2 Blood Hemolysis 30

3.4.3. Observation And Classification Of Colony Morphology 30

3.4.4. Phenotypic Detection Of Biofilm Formation 31

3.4.5. Biofilm Formation And Biomass Quantification 31

3.4.6. Composition Of The Extracellular Polymeric Matrix 31

3.5. Dissemination Of Antibiotic Resistance In Polymicrobial Communities 32

3.5.1. Mixed Biofilm Formation 32

3.5.2. Counting The Number Of Viable Cells 32

3.5.4. Dissemination Of Antibiotic Resistance To *P. aeruginosa* PAO1 33
3.5.5. Mixed Biofilm Resistance To Ceftazidime 33
3.6. Statistical Analysis 33
Chapter 4. Results And Discussion 34
4.1. Susceptibility Profile Of *Klebsiella pneumoniae* Isolates 34
4.2. Characterization Of The Virulence Potential Of *Klebsiella pneumoniae* Isolates 38
4.2.1. Hypermucoviscosity And Blood Hemolysis 38
4.2.2. Clonal Diversity 39
4.2.3. Phenotypic Detection Of Biofilm Formation 43
4.3. Biofilm Formation 45
4.3.1. Composition Of The Extracellular Polymeric Matrix 46
4.3.2. Biofilm Formation And Antimicrobial Resistance 49
4.3.3. Biofilm Formation And Clonal Diversity 50
4.4. Dissemination Of Antibiotic Resistance In Polymicrobial Communities 52
4.4.1. Dissemination Of Resistance To *P. aeruginosa* In 24-H Old Mixed Biofilms 52
4.4.2. Dissemination Of Resistance To *P. aeruginosa* In 72-H Old Mixed Biofilms 55
4.4.3. Mixed Biofilm Resistance Against Ceftazidime 57
5. Conclusions 61
References 63
Appendix 75

Abbreviations

A. baumannii	*Acinetobacter baumannii*
ARG	Antibiotic Resistance genes
CAZ	Ceftazidime
CDC	Center of Diseases control
CFU	Colony forming units
CV	Crystal Violet
CLSI	Clinical and Laboratorial Standards Institute
CR-KP	*Klebsiella pneumoniae* carbapenem-resistant
CRE	Carbapenem-resistant *Enterobacteriaceae*
CRE-KP	*Klebsiella pneumoniae* carbapenem-resistant *Enterobacteriaceae*
DNA	Deoxyribonucleic acid
E. coli	*Echerichia coli*
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended spectrum β-lactamases
ESBL - KP	*Klebsiella pneumoniae* extended spectrum β-lactamases producers
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species
hv-KP	Hypervirulent *Klebsiella pneumoniae*
K. pneumoniae	*Klebsiella pneumoniae*
LB medium	Luria Bertani medium
LPS	Lipopolyssacharides
MDDST	Modified Double Disc Synergy Test
MDR-KP	Multi-drug Resistant *Klebsiella pneumoniae*
mm	Millimeters
NCCLS	National Committee of Clinical Laboratory Standards
P. aeruginosa	*Pseudomonas aeruginosa*
P. mirabillis	*Proteus mirabillis*
PBP	Penicillin Bind Protein
PCR	Polymerase Chain Reaction

RNA	Rybonucleic acid
rpm	Rotations per minute
S. aureus	*Staphylococcus aureus*
SCV	Small Colony Variants
WHO	World Health Organization
UV	Ultraviolet
V. cholerae	*Vibrio cholerae*
XDR-KP	Extremely-drug Resistant *Klebsiella pneumoniae*

List of figures

Figure 1. Mechanisms of antibiotic resistance *in K. pneumoniae*. Adapted from Allen at al.,2010.....4

Figure 2. Horizontal gene transference between strains of the same species or between stains of different species or genera. Adapted from Furuya et al., 2006.....7

Figure 3. Representation of the four main virulence factors in classical and hypervirulent *K. pneumoniae*: capsule, LPS, fimbriae type 1 and 3 and siderophores. Adapted from Paczosa et al., 2016.....16

Figure 4. Stages of biofilm development. Adapted from Otto, 2009.....21

Figure 5. Phenotypic detection of ESBL production by *K. pneumoniae* clinical isolates using double disc synergism test.....37

Figure 6. Morphologic features of planktonic *K. pneumoniae* colonies observed on TSA, at 24 hours of incubation and the colony morphology development of the variants after 45 hours of growth.....40

Figure 7. Appearance of colony morphology of *K. pneumoniae* clinical isolates growth in a planktonic culture for 45 hours and plated in TSA.....43

Figure 8. Biofilm formation capacity of *K. pneumoniae* clinical isolates and ATCC 1129.....45

Figure 9. Biofilm biomass after enzymatic digestion of *K. pneumoniae* 24h-old biofilms in order to infer part of extracellular polymeric matrix.....47

Figure 10. A) Graphical representation of the biofilm formation capacity of PAO1, *K. pneumoniae* isolates, *K. pneumoniae* isolates in consortia with PAO1, for 24 hours. B) Counting of the number of viable cells from the mixed biofilms of *K. pneumoniae* strains and PAO1, in $\log_{10}$ CFUs/mL.....53

Figure 11. Dissemination of resistance from *K. pneumoniae* (Isolate 70) to PAO1.....54

Figure 12. Number of viable dual-species biofilm-cells of *K. pneumoniae* strains and PAO1, in $\log_{10}$ CFUs/mL.....55

Figure 13. Dissemination of resistance from *K. pneumoniae* (Isolate 94) to PAO1.....56

Figure 14. Number of cultivable dual-species biofilm-cells of *K. pneumoniae* strains and PAO1, in $\log_{10}$ CFUs/mL.....56

Figure 15. A) Graphical representation of the biofilm formation capacity of PAO1 without any treatment, PAO1 treated with 2 mg/L of CAZ and PAO1 treated with 8 mg/L of CAZ for 24 hours. B) Cells from PAO1

biofilms without any treatment, PAO1 treated with 2 mg/L of CAZ and PAO1 treated with 8 mg/L of CAZ for 24 hours.....58
Figure 16. Biofilm biomass of 24-h old bioiflms of K. pneumoniae and P. aeruginosa, with and without CAZ treatment for 24 h.....59
Figure 17. Number of cultivable dual-species biofilm-cells of *K. pneumoniae* strains and PAO1, in log_{10} CFUs/mL.....59
Figure 4.1. Results from the Congo red agar method used to determinate the biofilm formation capacity of *K. pneumoniae* clinical isolates and *K. pneumoniae* ATCC 11296.....79
Figure 4.2- Boxplots that display biofilm formation data for ESBLs production. The black bars in the boxes represent the median values.....79
Figure 4.3- Boxplots that display biofilm formation data for the presence of different resistance genes. A) SHV; B) TEM; C) CTX; D) KPC. The black bars in the boxes represent the median values.....80
Figure 4.4- Boxplots that display biofilm formation data for the presence of different double combinations of resistance genes. A) SHVCTX; B) SHVTEM; C) KPCTEM; D) KPCSHV. The black bars in the boxes represent the median values.....80
Figure 4.5- Boxplots that display biofilm formation data for the presence of different triple combinations of resistance genes. A) SHVTEMCTX; B) KPCSHVTEM. The black bars in the boxes represent the median values.....81
Figure 4.6- Boxplots that display biofilm formation data for the diversity of morphotypes. The black bars in the boxes represent the median values.....81
Figure 4.7- Boxplots that display biofilm formation data for the presence or absence of different types of margin in the colonies. A) Entire; B) Lobate; C) Undulated. The black bars in the boxes represent the median values.....82
Figure 4.8- Boxplots that display biofilm formation data for the presence or absence of different types of opacity in the colonies. A) Opaque; B) Translucent; C) Opaque and Translucent. The black bars in the boxes represent the median values.....82

List of Tables

Table 1. Information concerning the strains used throughout this study regarding their isolation site and susceptibility profile.....28

Table 2. Results of the antimicrobial susceptibility test of 9 clinical isolates of *K. pneumoniae* and *K. pneumoniae* ATCC 11296 used as a control strain.....35

Table 3. Results from the Congo red agar method used to determinate the biofilm formation capacity of *K. pneumoniae* clinical isolates and *K. pneumoniae* ATCC 11296.....44

Table 4. Differences between ESBL-producing and non-producing strains of *K. pneumoniae* in the capacity of biofilm formation, and the differences between the strains containing different resistance genes, according the Kruskal-Wallis test.....49

Table 5. Differences in biofilm formation between the isolates with double combinations of resistance genes, according Kruskal-Wallis test.....50

Table 6. Differences in biofilm formation between the isolates with triple combinations of resistance genes, according Kruskal-Wallis test.....50

Table 7. Differences between the number of morphotypes and the ability to form biofilm of *K. pneumoniae* strains, according to Kruskal-Wallis test.....51

Table 8. Differences in biofilm formation capacity between the *K. pneumoniae* strains with different colony characteristics, using Kruskal-Wallis test.....51

Table 3.1. Morphological features used to characterize *K. pneumoniae* colonies.....75

Table 4.2. Description of morphological characteristics observed at 24 and 45 hours of incubation.....76

Table 4.3. Detailed morphological description of the colonies identified in the clinical isolates of *K. pneumoniae*.....77

Chapter 1. Introduction

1.1. Scope of the thesis

The increasing and continuing acquisition of antibiotic resistance by *Klebsiella pneumoniae* is a major concern and requires a global commitment to halt this MDR pathogen. *K. pneumoniae* has become so important due to the emergence of strains producing ESBLs and carbapenemases that make it difficult the treat of infections leading to a significant increase in mortality and morbidity rates. Furthermore, the spread of these strains worldwide has been overwhelming, especially due to the emergence of hypervirulent strains capable of hosting healthy individuals. Given the current situation, it is increasingly important to understand in depth the relationship between the factors that lead to the spread of MDR strains and their acquisition / spread of resistance between species, which will provide answers to the development of new antimicrobial agents and changing the actual antimicrobial stewardship paradigm.

1.2. Objective

The objective of this work consisted in the investigation of the ecology and physiology of *K. pneumoniae* biofilms and understand how they provide antibiotic tolerance to bacteria to further use this knowledge to formulate effective antibiotic strategies and plan proper hospital infection control strategies to prevent the spread of *K. pneumoniae*.

1.3. General Outline

This thesis is structured in five chapters. This Chapter 1 intends to address the motivation for the theme of this study, describe the general objectives, as well as how it was organized. Chapter 2 presents the theoretical background on the impact of infections caused by multi-resistant strains of *K. pneumoniae* and the pathogenicity factors influencing their spread worldwide. Chapter 3 describes the experimental work performed, referring to the microorganisms, materials and procedures used throughout the work. Chapter 4 describes the results obtained and their discussion. Finally, Chapter 5 summarizes the main conclusions resulting from the experimental work and suggestions for future work.

Chapter 2. State of the Art

2.1. *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, encapsulated, rod-shaped and non-motile bacterium with 0,5 to 5 μm of length that belongs to the Enterobacteriaceae family. Enterobacteriaceae family is a very ample group of Gram-negative bacteria usually referred as enteric because the majority of them belong to the normal intestinal microbiota, such as *K. pneumoniae*. However, this family also includes a variety of other non-intestinal pathogenic bacteria[1].

K. pneumoniae is considered a facultative anaerobic, capable of respiratory and fermentative metabolism, lactose-fermenting, catalase positive but oxidase-negative microrganism[1]. This bacterium can be commonly found in the nature including surface water, soil and plants, and it is considered one of the most dangerous human pathogen accounting for about one third of all Gram-negative infections[2]. *K. pneumoniae* can typically cause a large spectrum of community-acquired and nosocomial infections on patients with a compromised immune system[3-5], such as pneumonia, urinary and biliary tract infections, gastrointestinal diseases, bacteremia, liver abscesses, osteomyelitis and endophthalmitis[6,7]. The development of these infections is facilitated if patients had some health risk condition, including advanced age, diabetes or previous use of antibiotics, such as metronidazole or glycopeptides[8]. However, some strains of *K. pneumoniae* are very propense to spread, making it a high virulent pathogen and able to cause infection in healthy individuals with no hospital historical[6,7]. In these cases pyogenic liver abscess, endophthalmitis, meningitis and bloodstream infections are the most common clinical situations observed[9]. This infection success on non-immunocompromised or ill patients is alarming and represents a great concern for medical and scientific community.

Over the years, *K. pneumoniae* has won clinical relevance because of the high prevalence of their infections ranging from 6 to 50 % across different countries and continents. In Europe the prevalence of infections varies substantially with geography, with most countries having a percentage close to the global minimum, while Greece has a prevalence of 50 % followed by Italy with about 29 % of infections caused by *K. pneumoniae*. Furthermore, the spread of infections among the population is extremely fast. In Italy, the second country in Europe with the highest prevalence of infections, registered an increase of 18 % in just 3 years[10].

The high prevalence of infections caused by *K. pneumoniae* and the worrying situation of hospital control of those, places this bacterium in the ESKAPE group. ESKAPE is the acronym of 6 bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas*

aeruginosa, and *Enterobacter* species) responsible for nosocomial infections with risk of death among critically ill and immunocompromised patients, due their resistance mechanisms against antimicrobial agents[11].

The spread of *K. pneumoniae* is a challenging public health threat as well as the application of infection prevention and control measures due to its virulence factors expression, hypervirulent phenotypes and remarkable antibiotic resistance mechanisms, which led to that *K. pneumoniae* infections becoming progressively more difficult to treat, making fundamental the understanding of the new paradigms in pathogenesis, transmission and resistance of this bacterium.

2.2. Antibiotic resistance

K. pneumoniae antibiotic resistance is currently a hot topic in clinical community worldwide[12], once often *K. pneumoniae* often shows a high resistance to a broad spectrum of drugs including β-lactam antibiotics, fluoroquinolones and aminoglycosides[13], multi-drug resistance (MDR-KP) and even extremely drug resistance (XDR-KP)[2]. MDR-KP causative infections are associated with increased mortality and morbidity rates due to the lack of effective treatments[10,14]. Nowadays this scenario represents so serious public health issue that MDR-KP is now considered by WHO a critical priority for the development of effective therapeutic interventions[15].

Over the years, bacteria developed adaptative resistance mechanisms against antimicrobial agents, since they were essentially molecules naturally produced in the same environment in which bacteria were inserted, and lately, due the excessive use of antimicrobial agents. The coexistence of bacteria and antimicrobial compounds led bacteria to use mechanisms to escape to antibiotic action. Indeed, the adaptive evolution of bacteria triggered the development of several mechanisms of action against antimicrobial agents, such as the alteration of the outer membrane permeability, efflux pumps, mutations that alter the active site of antibiotic binding, and genes encoding antibiotic-destroying enzymes[15,16] (Figure 1).

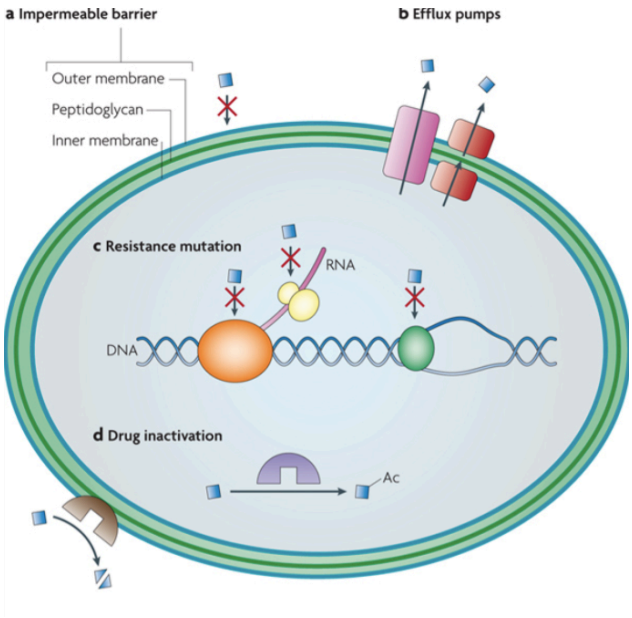


Figure 1. Mechanisms of antibiotic resistance *in K. pneumoniae* a) Impermeable barriers. Some bacteria are intrinsically resistant to certain antibiotics, others can alter the permeability of their membrane in order to resist the antibacterial action. b) Efflux pumps secrete antibiotics from the cell. Some transporters can pump the antibiotics directly outside the cell (pink) and others secrete them into the periplasm. c) Resistance mutations modify the target protein. d) The inactivation of the antibiotic occurs by modification of the drug thought the action of enzymes. (Adapted from Allen et al., 2010)[164].

In gram-negative bacteria, such as *K. pneumoniae,* the target site for the action of antimicrobial agents (PBP – Penicillin Binding Protein) is found in the cytoplasmic membrane and antibiotics must penetrate the outer membrane in order to reach it and act effectively. However, bacteria developed a mechanism of the outer membrane permeability that leads to a decrease of the cellular input of external substances, acting as a line of first defense against the penetration of toxic compounds, including antimicrobial agents avoiding thus their toxic effect on them. This mechanism essentially affects hydrophilic molecules such as β-lactams, tetracyclines and some fluoroquinolones, in this way, these molecules began to alternatively use porins to cross the membrane and reach PBP[17].

Nevertheless, porins may also interfere with antibiotic resistance by alteration of the porin type expression, increase/decrease of its expression or by impairment of its function[18]. Clinical isolates of *K. pneumoniae* were collected before and after treatment with β-lactam antibiotics and the porin expression was evaluated for the porin expression. In the clinical isolates collected post-therapy, there was a modification in the type of expressed porin, being that the isolates started to express a type of porin with a smaller channel in order to prevent penetration of the antimicrobial agent into the interior[19].

Efflux pumps represent a machinery capable of externalizing components that are considered toxic or inhibitory to bacterial activity after their uptake, preventing the components action over the cell target sites.

This system may be substrate specific or with broad substrate specificity, the latter is usually found in MDR bacteria such *K. pneumoniae*. Efflux pumps affects a wide range of antimicrobial classes including protein synthesis inhibitors, fluoroquinolones, β-lactams, carbapenems and polymyxins[20]. The genes encoding this mechanism can be found in mobile genetic elements or in the chromosome, the last ones can explain the intrinsic resistance of some bacteria to certain antibiotics[20].

The efflux pumps are divided by different families that are distinguished in terms of structural conformation, energy source, range of substrates on which they can act and in the type of bacteria in which they are found. The main 5 families include *i)* the major facilitator superfamily (MFS), *ii)* the small multidrug resistance family (SMR), *iii)* the resistance-nodulation-cell- division family (RND), *iv*) the ATP-binding cassette family (ABC), and *v*) the multidrug and toxic compound extrusion family (MATE)[21].

An example of efflux-mediated resistance is the activity of Tet efflux pumps that extrude tetracyclines through proton exchange as a source of energy. The most part of *tet* genes has been described as belonging to mobile genetic elements[22]. In addition to specific systems for expulsion of tetracycline, there are several MDR efflux pumps capable of expelling tetracyclines, which contributes to multidrug resistance[23]. Furthermore, MDR pumps belonging to the RND family are often found on the chromosome of gram-negative bacteria with clinical relevance and determine the intrinsic resistance to various antibiotics[22].

One of the most common mechanisms of *K. pneumoniae* antibiotic resistance includes the modification on the target site for the antibiotic with the aim of weakening the affinity between them, this type of mechanism affects almost all classes of antimicrobial compounds including β-lactams, glycopeptides, macrolides, lincosamides, and streptogramins (MLS), and aminoglycosides. These modifications can occur by point mutations in the genes encoding the target site, enzymatic alterations on the binding site and by replacement of the original target[24].

The acquisition of resistance to antibiotics through genetic mutations is exemplified by resistance to rifampicin that inhibits DNA-dependent RNA polymerase in order to block bacterial transcription[25]. Another example of mutational resistance is related to resistance to fluoroquinolones. The mechanism of action of these antibiotics is to alter DNA replication through inhibition of DNA gyrase and topoisomerase IV, mutation in the genes encoding the subunits of these enzymes is the most frequent cause of acquired resistance to these compounds[26].

Resistance through enzymatic modification of the target site occurs, for example, in resistance to macrolides, antibiotics which act as inhibitors of bacterial protein synthesis. This mechanism consists of the methylation of the ribosome catalyzed by an enzyme encoded by the erm genes, which impairs the binding of the antibiotic to its target site[27].

Another strategy involves replacing the target site, where bacteria acquire the ability to find new targets with similar functions to the original target but are not inhibited by the antimicrobial agent. One of the most relevant examples is methicillin (β-lactam) resistance in *S. aureus* due to the acquisition of an exogenous PBP. In addition, the antimicrobial action may be inhibited by bacteria by altering the metabolic pathway on which the antimicrobial agents interfere, an example being resistance to trimethoprim-sulfamethoxazole[28].

The most successful strategy used by bacteria to resist the action of antimicrobials is the production of enzymes capable of inducing chemical changes in the molecule of antibiotic leading to its inactivation or destruction. Some examples are *β*-lactamases, aminoglycoside-modifying enzymes, or chloramphenicol acetyltransferases[14]. The destruction of the antibiotic molecule is a typical mechanism of β-lactam resistance, in which the produced β-lactamases enzymes have the capacity to destroy the amide bond of the β-lactam ring. Genes encoding for β-lactamases are included in the *K. pneumoniae* resistome, which consists of all the genes that confer resistance to the bacterium[2]. These genes are generally found in the chromosome or localized in mobile genetic elements as part of the accessory genome, accordingly, β-lactam resistance can have an intrinsic or acquired origin[29].

The production of β-lactamases, notably the extended-spectrum β-lactamases (ESBL) puts *K. pneumoniae* as one of major contributors for the current global crisis of antibiotic resistance. ESBL-producing *K. pneumoniae* (ESBL-KP) can resist to penicillins, first-, second-, and third-generation cephalosporins and aztreonam, which become the treatment of *K. pneumoniae* - associated infections a critical situation[2]. The dissemination of these strains led to an increased use of last-line β-lactam antibiotics, the carbapenems, but since the early 2000s, carbapenem-resistant *K. pneumoniae* (CR-KP) was detected due to the production of carbapenemases[10,30]. These enzymes represent the most versatile and highly transmissible β-lactamases being now found throughout the world[31].

Alongside the resistome that can evolve and can accumulate various antibiotic resistance genes leading to the evolution to MDR phenotype, acquisition of plasmids carrying antibiotic resistance genes, including ESBL- and carbapenemase-encoding genes have demonstrated to be a crucial mechanism for rapid resistance expression and dissemination. These transferrable plasmids are referred to as *K. pneumoniae* mobilome[2]. Normally, the acquisition of foreign DNA happens thought three different forms: transformation, transduction and conjugation (Figure 2).

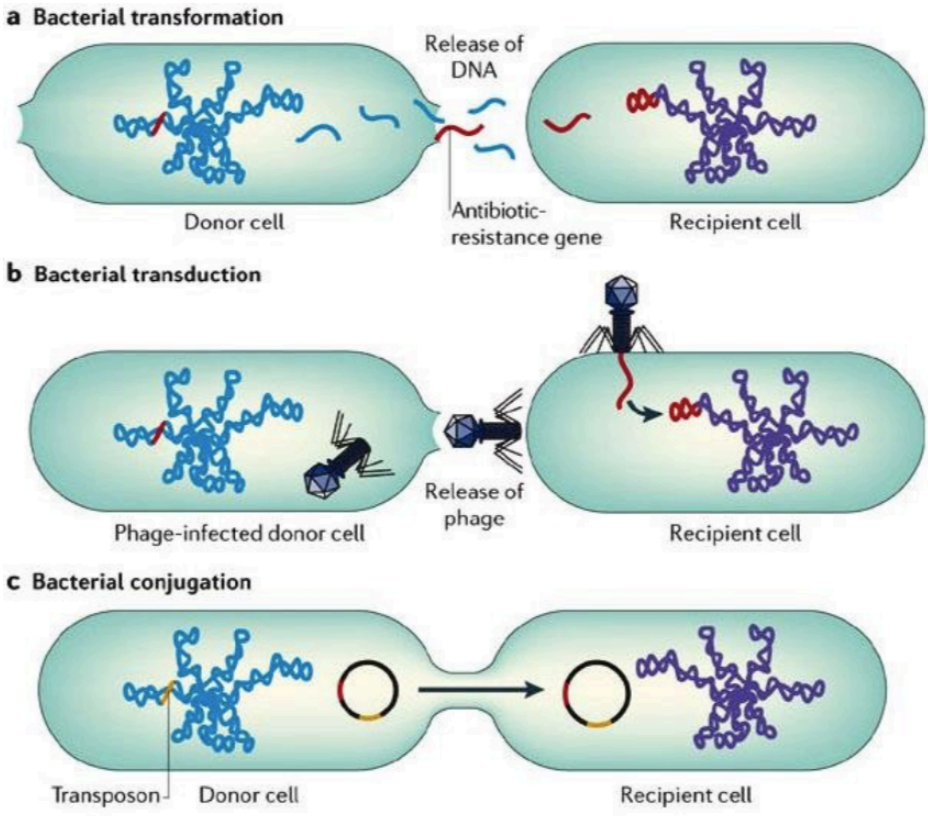


Figure 2- Horizontal gene transfer between strains of the same species or between strains of different species or genera. a) Transformation occurs when naked DNA is released on lysis of an organism and is taken up by another organism. b) In transduction antibiotic-resistance genes can be integrated into the chromosome of the recipient cell. c) Conjugation occurs by direct contact; plasmids form a mating bridge across the bacteria and DNA is exchanged, which can result in acquisition of antibiotic-resistance genes by the recipient cell. (Adapted from Furuya et al., 2006) [165].

Transformation is probable the simplest mechanism of horizontal gene transference and involves the internalization and chromosomal integration of DNA on the bacterial genome[32] (Figure 2.a). About 80 bacterial species use this mechanism, and are for that reason, classified as naturally transformable[2,33]. For the transformation to occur the bacteria need to be found in the state of competence, which consists of a very regulated physiological state in which a specific set of proteins is expressed, this set of proteins is called transformasome. The transformasome proteins have the function of allowing the binding and internalization of extracellular DNA in the genome, being its role fundamental in the transformation[33]. Although it is a relatively simple method of horizontal gene transfer, transformation occurs naturally in only a few clinically relevant species[15].

Transduction consists on bacteriophage-mediated transfer of DNA from a donor cell to a recipient cell (Figure 2.b)[34]. Essentially, bacteriophages are characterized in two forms, in lysogenic or lytic phages. The first can infect the bacteria and live within them without any interruption in the normal cycle of the bacterial cell, the viral DNA is incorporated into the genome of the host cell and this continues to reproduce itself normally transmitting the virus to the subsequent generations. The lytic phages insert their DNA into

the host cell, dominating their metabolism and leading to the rupture of the same at the end of the process. After cell rupture, the phage particles that carry the host genome are released to be able to infect new cells and thus transmit important genetic information regarding antibiotic resistance and pathogenicity factors[35]. However, the importance of bacteriophages in the horizontal transfer of genes is undervalued when compared with other transfer pathways, such as conjugation[34].

Conjugation is the main mechanism for the acquisition of antibiotic resistance genes by bacteria and responsible for the emergence of resistance and its dissemination in hospital environment, since it is a very efficient path of gene transfer and tends to occur at high rates in the gastrointestinal tract of patients under antibiotic treatment [15]. Alike the above mechanisms, the conjugation occurs through the transfer of genetic material between a donor cell and a recipient cell. However, in conjugation, the transfer of genetic material between the cells occurs through direct contact between them and it is necessary to ensure the mobilization of DNA and the formation of mating pairs (Figure 2.c)[36]. Conjugation usually uses mobile genetic elements for sharing of genetic information, although it may also occur, although less frequently, through integrative conjugative elements inserted into the chromosome. The most important mobile genetic elements are plasmids and transposons, since they are mainly responsible for the development and dissemination of resistance to antibiotics among clinically relevant organisms such as *K. pneumoniae*[15].

Currently, the existing problem in clinical settings on antibiotic resistance does not refer to the prevalence of bacteria with intrinsic resistance, but rather to the expression of acquired resistance in bacteria that were initially susceptible to the action of certain antimicrobial agents[37]. *K. pneumoniae* have been enriching their mobilome with genes encoding for multiple antibiotic resistance mechanisms, including the production of ESBLs, *Amp*C β-lactamases and carbapenemases[38]. Due to their clinical relevance ESBL-producing bacteria and carbapenem-resistant *K. pneumoniae* (CR-KP) will be next reviewed in detail.

2.2.1. Extended Spectrum β-Lactamase-producing *Klebsiella pneumoniae*

The inappropriate and extensive use of β-lactam antibiotics to treat *K. pneumoniae*-associated infections has led to the emergence of bacterial strains capable of producing β-lactamases able to destroy the β-lactam antibiotic ring in the cell. In *K. pneumoniae,* these enzymes are secreted into the periplasmic space, that together with the reduced permeability barrier of the outer cell wall, resulting in clinically significant resistance to antimicrobials[39].

β-lactams are the most prescribed antibiotic in clinical practice and since their introduction in human medicine hundreds of different β-lactamases have evolved. In 1940 was discovered the first gram-negative chromosomally encoded β-lactamase in an *Escherichia coli* strain[40]. After 25 years, the first plasmid-encoded

β-lactamase, TEM-1, was identified also in *E. coli* and shortly after a sulfhydryl variant of TEM termed SHV-1 able to hydrolyze a larger spectrum of β-lactam antibiotics, including oxyimino-cephalosporins appeared in *Klebsiella,* as well as, in *E. coli*. These features led to these enzymes being designated as ESBLs[2,40]. In general, ESBLs are β-lactamases that confer resistance to bacteria against all β-lactams, except for carbapenems and cephamycin, including penicillins, the first-, second-, and third-generation cephalosporins, and to aztreonam by hydrolyzing these antibiotics, and their activity is repressed by β-lactamase inhibitors, such as clavulanic acid[41,42]. Since ESBL discovery, several other ESBL variants have been reported in *K. pneumoniae*, becoming the major ESBL-carrying pathogen associated in nosocomial outbreaks[41].

The number of ESBL-associated infections has increased reaching an astonishing prevalence worldwide with proliferation even in countries where standards for the use of antibiotics are restricted[43]. Moreover, the rapid spread of ESBL-producing strains to other gram-negative bacteria, including other Enterobacteriaceae and also *Acinetobacter* and *Pseudomonas* led to *K. pneumoniae* becomes one of the major concerns in hospital infections, especially due to the clinical outcomes and limited antibiotic treatment options[37,42].

ESBLs are commonly classified according to their amino acid sequence homology based on the scheme devised by Ambler *at al.*[39], this scheme classifies β-lactamases into four different classes. Class A, C and D enzymes are characterized by an active-site serine and for that reason are serine β-lactamases and class B enzymes are metallo-β-lactamases[44]. Class A enzymes form a heterogeneous molecular group, which comprises a wide range of proteins with different catholic activities, including the most commonly found ESBLs, the SHV, TEM and CTX-M[45]. Enzymes belonging to this class are normally encoded in plasmids but may also pertain to the bacterial chromosome for example the SHV encoding gene, bla_{SHV}, which is embedded in the bacterial chromosome of *K. pneumoniae* conferring intrinsic resistance to ampicillin, can also be found in transferable plasmids[39]. The bla_{SHV} gene has evolved as a chromosomal gene in *K. pneumoniae* and subsequently incorporated in a plasmid that has spread among other species of enterobacteria, such as *E. coli*, being part of the conjugative plasmid p453, favoring the spread of resistance to penicillins such as ampicillin, tigecycline and piperacillin[44,46]. Although SHV enzymes were initially identified in *K. pneumoniae* and *E. coli*, their presence in other Enterobacteriaceae species, with superior allelic variability and in different environments, has been observed in the last years, which recalls the importance of the dissemination of plasmid-associated SHV β-lactamases and their implication at the epidemiological level[47]. The genetic variability found in these enzymes allowed the detection of SHV capable of inferring resistance to third generation cephalosporins, monobactams and carbapenems, raising the resistance profile

of the species that contain them, worsening the scenario of antibiotic resistance that is currently verified at a global level[46]. SHV β-lactamases are currently divided into three fundamental groups that characterize them by their functional properties. The first group encompasses the enzymes responsible for the hydrolysis of penicillins and early cephalosporins and are strongly inhibited in the presence of clavulanic acid and tazobactam. The SHVs of the second group have a broad-spectrum action and are perceptible by the resistance they have acquired to clavulanic acid. Finally, the last group corresponds to enzymes capable of hydrolyzing one or more of the following antibiotics: cefotaxime, ceftazidime and aztreonam[46].

Similarly, TEM β-lactamases have, over time, undergone some changes at the molecular level which allowed the detection of new TEM derivatives. These enzymes may undergo changes in the amino acid sequence altering the phenotype, thus the TEM derivatives may be extended- spectrum or inhibitor-resistant β-lactamases. Some of these β-lactamases are inhibitor-resistant, however the majority are ESBLs. Changes occurring in the amino acid sequence in the enzyme have a limited number of positions and may result in alteration of the phenotype in the sense of inferring ability to hydrolyze oxyimino-cephalosporins, such as ceftazidime or cefotaxime, or change the isoelectric point of the enzyme[47]. TEM-1 is the most common encoded β-lactamase among gram-negative bacteria and is able to hydrolyze penicillins and first-generation cephalosporins. Its first derivative, TEM-2, arose due to the alteration of an amino acid in the original enzyme which caused a change in the isoelectric point but did not alter the substrate profile. The first β-lactamase with ESBL phenotype was the TEM-3 enzyme[16,48]. Although TEM type ESBLs are currently present in other species, such as in *K. pneumoniae*, the major producer of bla_{TEM} genes is *E. coli*. The transmission of these to other species presupposes the mutation of the genes bla_{TEM-1} and bla_{TEM-2} and occurs through plasmids[42].
The presence of ESBLs of type SHV and TEM was frequently observed until the early 1990s, with the majority of ESBL-associated infections being due to the clonal spread of SHV-producing *K. pneumoniae* at the hospital level[42]. Currently, the spread of new strains of ESBLs from the family SHV and TEM are emerging in Europe, where there has been reported nosocomial infections caused by *Salmonella* with TEM-52 in Spain[16].

The most recent β-lactamase family is CTX[49]. Although CTX enzymes are the most recent discovered type of β-lactamases, they have been reported as the genetic family most associated to resistance[45,47] and with a high clinical impact. This resistance family did not arise from alterations in existing enzymes, but through horizontal gene transfer[45], since this is a plasmid-encoded ESBL. These mobile elements can be apprehended by a broad range of conjugative plasmids or phage-like sequences that can serve as vehicles for dissemination. Subsequently, CTX enzymes have become the most prevalent ESBL worldwide and also responsible for the cephalosporin resistance in *E. coli* and *K. pneumoniae*[15]. CTX-M β-lactamases are characterized by rapidly acting on the hydrolysis of cefotaxime and for being more easily inhibited by the

β-lactamase inhibitor tazobactam than by sulbactam and clavulanate[16]. The CTX family presents a low homology with the remaining resistance families of ESBLs, having only 40 % of identity with TEM and SHV[47]. Currently, there are numerous CTX enzyme variants that are grouped into five distinct groups (CTX-M group 1, 2, 8, 9, and 25) according to their amino acid sequence[16].

The dissemination of bacterial strains capable of producing CTX-M β-lactamases is increasingly worrying, since it does not occur only in the hospital environment. In addition, they are rapidly emerging and the acquisition of genes encoding CTX-M enzymes, bla_{CTX-M}, currently occurs in the community. Additionally, the dissemination of ESBLs also occurs through animals used for food and/or as pets, suggesting that bla_{CTX-M} ESBL genes detected in pathogenic bacteria are acquired from environmental bacteria[42].The prevalence of infections triggered CTX-M ESLBs is usually higher in the Eastern Europe[45], however recent studies point to the prevalence of CTX-associated infections, also in South Europe[50]. In Asia, investigations point to a higher incidence of CTX in infections over other families of ESBLs. In this case, the situation is more alarming, since the Asian countries have high prevalence rates of ESBLs[45].

As noted above, the most common ESBLs associated with infections and clinically isolated are SHV, TEM and CTX-M, however ESBLs are not confined to class A β-lactamases, as such, various oxacillinases are also considered to be extended spectrum and designated as OXA ESBLs. In addition, other types of ESBLs, as SFO, BES, BEL, TLA, GES, PER, and VEB have also been reported[43]. These are considered rare due their low prevalence and little knowledge about them, such that these enzymes do not derive from any known β-lactamases and, with the exception of SFO, their progenitor genes remain unknown[51]. So far is known that bla_{SFO}, bla_{BES}, bla_{BEL} and bla_{TLA} genes are very rare and geographically localized, while the bla_{GES}, bla_{PER} and bla_{VEB} genes have been described in several continents[51].

2.2.2. Carbapenem-resistant *Klebsiella pneumoniae*

The endemic occurrence of ESBL-producing *K. pneumoniae* leads to a significant use of carbapenems, since it is the last resort to treat their causative infections. Unfortunately, since the early 2000s, its extensive use of led the emergence of carbapenem-resistant *K. pneumoniae* (CR-KP) able to the produce carbapenemases. Carbapenemases can inactivate carbapenems and hydrolyze all β-lactam antibiotics such as penicillins, cephalosporins and monobactam, including the last-line carbapenems. These enzymes represent the most versatile and highly transmissible β-lactamases being now found over the world[31]. Carbapenemases have been described in Enterobacteriaceae and according to the European CDC report, *K. pneumoniae* is the most common carbapenem-resistant *Enterobacteriaceae* (CRE)[52]. Also, other Gram-negative bacteria such as *Acinetobacter baumannii*, *Proteus mirabilis* and *P. aeruginosa* can exhibit

carbapenem resistance, posing a significant public health theat[15,53]. A study carried out by the European CDC showed that 1,3 patients out of every 10000 hospital admissions had an associated-infection with carbapenemase-producing *K. pneumoniae* or *E. coli*, with the highest incidence found in southern and southeastern Europe[54]. *K. pneumoniae* strains resistant to carbapenemases are increasingly common, observing a significant increase in several countries since 2005. Examples are Italy, Israel, Greece and Portugal that present a high rate of clinical isolates carbapenemases-producers[52]. The presence of *K. pneumoniae* carbapenem-resistant *Enterobacteriaceae* (CRE-KP) have also been described in Asia[55]. Recent data from CDC demonstrated that of the 9000 infections due CRE, *Klebsiella* species were responsible for 80 % of the infections, in United States[56]. Generally, the mechanism under carbapenem resistance in *K. pneumoniae* involve the production of carbapenemases and the loss or decreased expression of outer membrane proteins[57].

CP-KP produce different types of carbapenemases, which like the remaining β-lactamases are functionally divided into distinct classes. The serine-dependent class A comprises the KPC family (*Klebsiella pneumoniae* Carbapenemase), class B metallo-β-lactamases includes the VIM (Verona Integronencoded metallo-β-lactamase), IMP (imipenemase metallo-β-lactamase) and NDM (New Delhi metallo-β-lactamase) families. Such as in class A, class D enzymes are serine-dependent and this class comprises the OXA-48 family (Oxacillinase-48)[58,59].

KPC is the most important carbapenemase at the clinical level and it was originally found in strains of *K. pneumoniae*[60]. KPC-1 emerged in 1996, in USA, and it has since been the most reported[8]. Since the appearance of KPC-producing strains, resistance to carbapenem evolved quickly, and the spread of KPC-producing *K. pneumoniae* strains becomes increasingly a matter of concern for public health. Globally, the prevalence of these strains is at lower levels, however, according to WHO there are countries like Iran and Greece with alarming rates above 50 %[10]. In the USA, the prevalence of KPC-producing bacteria varies greatly from state to state, however most reported cases concern a single species and type of strain, *K. pneumoniae* ST258[61].

K. pneumoniae with sequence type (ST) 258 is considered globally as a "high risk clone". This strain is highly associated with the development of the bla_{KPC-2} and bla_{KPC-3} genes through clonal expansion and transmission of clonal lineages that maintain the stable encoding the genes encoding carbapenemases. However, the horizontal transfer of carbapenemase genes through mobile genetic elements such as plasmids is primarily responsible for the spread of bla_{KPC-2} and bla_{KPC-3}[62]. Although these enzymes are mostly found in *K. pneumoniae*, KPCs are highly transferable to other organisms. They have been reported in several other Gram-negative bacteria, including *E. coli, P. mirabilis*, *Salmonella* spp. and *P. aeruginosa*[61].

CR-KP also produces oxacillinase (OXA-48), the second most prevalent carbapenemase detected, following the KPC[63]. The first OXA-48 β-lactamase was reported from a *K. pneumoniae* clinical isolate in Turkey, in 2001. This clinically significant enzyme is capable to hydrolyze penicillins and carbapenems, especially imipenem, but do not possess any activity against broad-spectrum cephalosporins, with the exception of having additional β-lactamase genes which allow to destroy these cephalosporins[64]. Besides that, OXA-48 enzymes are poorly inhibited by clavulanic acid[65]. OXA-48 β-lactamases are vastly disseminated in *K. pneumoniae* and in several Enterobacteriaceae, however they were also reported in *A. baumannii*[65]. The dissemination of this plasmid-encoded bla_{OXA-48} gene among species represents a very worrying scenario with regard to antibiotic resistance, especially to antibiotics used as the last defense line, which is the case of carbapenems, used to combat *K. pneumoniae*-associated infections[53,66]. These enzymes have a higher prevalence in Europe, especially in Mediterranean countries. However, OXA-48-like enzymes have been found in several countries in the Middle East, Africa, Asia and South America[65].

The genetic families VIM, IMP and NDM are metallo-β-lactamases once they use a metal ion as cofactor, instead of a serine residue, for the inactivation of the β-lactam ring. This enzymes are characterized by the ability to hydrolyze all β-lactams except aztreonam, by their resistance to all β-lactamase inhibitors and susceptibility to EDTA inhibition, since the mechanism of hydrolysis depends on the interaction of β-lactams with ions of zinc in the active center of the enzyme[64].

NDM was identified for the first time in Sweden in a patient with a previous hospitalization in New Delhi, this so named as "New Delhi". There are currently several variants of these enzymes, which differ from each other in the sequence of amino acids. These changes increase a diversity of enzyme types which contributes to difficulty in both detection and treatment[65]. The encoding gene of the β-lactamases type NDM, bla_{NDM}, is often carried by plasmids and thus is easily transported, through horizontal transfer of genes, to other microorganisms. Its presence has been reported in several types of plasmids such as IncA/C, IncF, IncN, IncL/M or IncR. The encoding of resistance genes in plasmids and other mobile genetic elements significantly increases the rate of dissemination of resistant strains among pathogenic microorganisms[63]. NDM-producing bacteria have been reported from many parts of the world, which shows the rapid spread of these β-lactamases, even faster than KPC spread[60].

The earliest *K. pneumoniae* strains producers of VIM were identified in southern Europe, but quickly spread to the Center Europe to countries such as Germany and France, and also to the USA. However, the prevalence of these enzymes in infections remains low and even decreasing in some countries[67].

The production of IMPs by *K. pneumoniae* was described for the first time in Asian countries and its dissemination into the rest of the world seems to be limited, with just some cases identified in Turkey,

Lebanon, Brazil and USA[67].

With the emergence of bacterial strains producing ESBLs and increasingly resistant to antibiotics, carbapenems began to be used in clinical practice as a last resort for the treatment of infections, however their widespread use culminated in the development of new resistant strains worldwide. The global spread of carbapenem-resistant *K. pneumoniae* has become a serious clinical challenge because of the limited treatment options[57].

2.2.3. Multiple β-lactamase-encoding *Klebsiella pneumoniae*

Along with its high prevalence, *K. pneumoniae* is one of the main sources of antibiotic resistance as the species continuous evolution allowed this bacterium to be able to increase its resistome, through the accumulating of several antimicrobial resistance genes (ARGs) that led to the emergence of MDR-KP and XDR-KP strains[2]. The worldwide increase of MDR and XDR *K. pneumoniae* is reflected in the spread of these strains through ARG transfer that can occur vertically (transmission to daughter cells) or horizontally, as is often referred to, by plasmids or transposons[68]. Due to their genetic diversity, *K. pneumoniae* also contributes to the spread of ARGs for other species or genera, benefiting from the fact that it is widely distributed among the environment, has a varied genetic identity and more plasmids than other Gram-negative bacteria[69].

The major problem associated with the spread of MDR and XDR *K. pneumoniae* are their ability to survive to treatments with a variety of antibiotics, that consists of the ability of this pathogen to have several β-lactam encoding genes (ESBLs and carbapenemases) in the same strain, forming such a complete resistome that it makes *K. pneumoniae* almost unbeatable. A resistome is an expression that defines the collection of all antibiotic resistance genes and their precursors in both non- and pathogenic bacteria. Presently, combinations of all resistance families have been reported in *K. pneumoniae* strains[70]. Also, a study carried out in Turkey allowed the detection of several antibiotic resistance genes within the same *K. pneumoniae* strain. In this case, the clinical isolates of *K. pneumoniae* had β-lactam resistance genes (bla_{CTX} and bla_{TEM}) and most of them carried carbapenem resistance genes (bla_{OXA-48}, $bla_{OXA-204}$ and bla_{NDM})[71]. In addition, there appears to be an interaction between different genetic families that alter the type of strain resistance. For example, carbapenemases of the IMP, VIM and NDM families are susceptible to aztreonam action, but in the presence of AmpC they become resistant to this antibiotic. Additionally, the presence of extra copies of the genes also appears to be related to an increase in bacterial resistance or response against an increase in antibiotic concentration[2].

The emergence of these super strains of *K. pneumoniae*, resistant to almost all available classes of antibiotics is a global concern due to limited treatment options and its early identification is essential for the control of infections in the hospital environment.

2.3. *Klebsiella pneumoniae* virulence factors expression

The outcome of an infection caused by a microbial pathogen is a function of the bacterial ability to develop infection, resist to clearance by host-defense mechanisms and/or by antibiotic killing[72]. Virulence factors are characteristics developed by pathogenic microorganisms that facilitates their colonization, invasion and infection development in the human host[73]. The most important virulence factors of *K. pneumoniae* include hypermucoviscosity and capsular polysaccharides, type 1 and 3 fimbriae, siderophores and factors involved in aggregative adhesions[3]. In general, *K. pneumoniae* infections are not a result of the production of a single virulence factors but rely on a battery of virulence determinants that facilitates the successful colonization and growth in the human host. By this reason, the various virulence factors expressed are strongly connected. The appearance of different phenotypes may be an example of mechanisms as horizontal gene transfer via plasmids, bacteriophage or transposons, which results in increased virulence strains[6]. One of the phenotypes that results in an increased of virulent factors, are the hypervirulent *K. pneumoniae* strains (Figure 3).

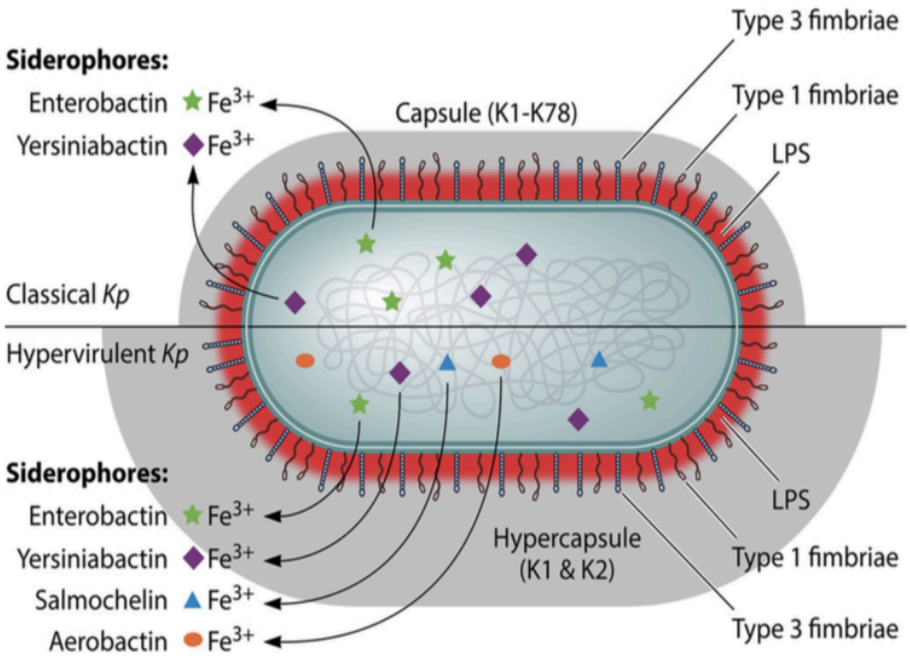


Figure 3- Representation of the four main virulence factors in classical and hypervirulent *K. pneumoniae:* capsule, LPS, fimbriae type 1 and type 3 and siderophores. Hypervirulent *K. pneumoniae* strains produce a capsule with serotypes K1 and K2 that are associated with increased pathogenicity. LPS is produced for both classical and HV-KP strains and can be of O-antigen serotypes 1 to 9. Both types of *K. pneumoniae* make membrane-bound adhesive structures, type 1 and type 3 fimbriae, and secret iron-scavenging siderophores. (Adapted from Paczosa et al., 2016) [82].

Hypervirulent *K. pneumoniae* emerge in Asian Pacific Rim, in the 1990's[74]. The infections were unique once they did not have the same origin from the infections with *K. pneumoniae* in hospitalized patients. Nowadays, in China, there are a high prevalence of virulent strains among *K. pneumoniae* isolates that causes infection, about 31 to 37,8 %, dependent on region[75]. Hypervirulent *K. pneumoniae* strains emerged from Asia, being represented in individuals of Asian descent, suggesting that the infection could be associated with individual's ethnicity or that is may be a geographically-specific pathogen[76]. Those strains have begun to emerge around the world, including United States[77].

2.3.1. Capsule

K. pneumoniae possesses a polysaccharide capsule that is an important factor for its pathogenesis because it provides protection against phagocytosis[78], and it is used to assist in evading the immune system of the host during infection. The capsule is formed by the capsular polysaccharide synthesis (cps), and it is a structure on the outside of the bacterial cell, attached to the exterior membrane[9]. There are several capsular serotypes, termed K-antigens, that are distinguished by the different chemical structure of the capsular

polysaccharide. In clinical isolates of *K. pneumoniae*, at least 79 serotypes have already been identified[79]. The various serotypes have been associated with different types and severity of infections caused by *K. pneumoniae*, and serotypes K1 and K2 are considered an important virulence factor of this species, partly and as mentioned above, for conferring resistance to the bactericidal activity mediated by phagocytes and inflammatory response and by inducing an increased antimicrobial resistance[6]. *K. pneumoniae* possessing K1 and K2 serotypes lack a mannose residue, preventing the recognition for the host factors, such as the mannose binding receptor on macrophage[80]. Moreover, K1 and K2 serotypes possess a specific recognition site on the surface that are host-specific, allowing evasion of the host immune cells[81]. Besides that, K1 and K2 strains may induce a smaller liberation of reactive oxygen species by neutrophils than other serotypes, ensuring better survival in human tissues[82].

The genes encoding capsule production in both common strains of *K. pneumoniae* and hv-KP are located on a chromosomal operon (cps) where the organization and sequence of the genes are conserved. This chromosomal operon harbors a variety of genes implicated in capsule production, including *wzi*, *wza*, *wzb*, *wzc*, *gnd*, *wca*, *cpsB*, *cpsG*, and *galF*[83]. The *wzi* locus is especially important since it is present in all types of *K. pneumoniae* capsule and allows the typing of K antigens, because the different sequences found are strongly associated with specific K-antigens[84].

Some strains of *K. pneumoniae* have the ability to produce a hypercapsule, which differs from the original in that it is more robust and consists of mucoviscous polysaccharides. Due to its structure, this hypercapsule gives extra protection by making *K. pneumoniae* strains much more virulent than common strains, they are called hypervirulent (hv-KP)[82]. In hv-KP strains the super production of capsule is essentially induced by the expression regulator gene of mucoid phenotype A (*rmp*A/ *rmp*A2), being this fact recognized by the high prevalence of genes *rmp*A/ *rmp*A2 up to 100 % in hv-KP strains, in contrast to 20 % in common strains of *K. pneumoniae*[85].

2.3.2. Type 1 and 3 Fimbriae

The adhesion of bacteria to host surfaces is considered an essential step in the development of infection and a very important virulence factor. Normally, bacterial adhesion is mediated by fimbriae, filamentous structures present on the bacterial cell surface. In *K. pneumoniae* type 1 and type 3 fimbriae are the most expressed and also the best characterized as pathogenicity factors[86].

Type 1 fimbriae have mannose residues as receptors and are responsible for adhesion to mannose-containing structures in host cells and in the extracellular matrix[87]. These adhesive structures consist of thin filaments on the bacterial surface and are present in almost all bacteria of the Enterobacteriaceae family. In

K. pneumoniae they are expressed in about 90 % of clinical isolates and environmental strains[82]. Type 1 fimbriae are encoded by the *fimABCDEFGHIK* gene cluster, the *fim*A gene encodes the major structural subunit (FimA), while the *fim*H gene encodes the adhesive subunit (FimH) that is located at the end of the filament[86]. Type 3 fimbriae have been shown to play an essential role in *K. pneumoniae* biofilm formation[87]. Type 3 fimbriae are helix-shaped filaments that, in *K. pneumoniae*, are codified by the *mrk*ABCDF gene cluster. The largest structural subunit (MrkA) is codified by the *mrk*A gene, while the adhesion subunit (MrkD) is encoded by the *mrk*D gene[86]. Beside *K. pneumoniae*, type 3 fimbriae are commonly found in *Citrobacter*, *Enterobacter*, *Serratia*, *Proteus*, and *Providencia* isolates and they have also recently been identified in clinical isolates of *E. coli*[86]. These fimbriae are responsible to the adhesion to several cell types *in vitro*, however, the specific receptor has not been found yet.

The expression of fimbriae by *K. pneumoniae* can be regulated according to the place of infection, especially in the expression of type 1 fimbriae in urinary tract infections. Interestingly, these are expressed in the urinary tract but not in infections of the gastrointestinal tract or lungs. This observation highlights the fact that type 1 fimbriae contribute to the invasion of bladder cells by *K. pneumoniae* and to biofilm formation during infection[82,86]. In addition, the bacterial growth form may also interfere with the expression of fimbriae in *K. pneumoniae*. It is known that in planktonic growth none of the types of fimbriae are expressed, whereas during biofilm formation type 3 fimbriae are expressed. This observation only emphasizes the importance of type 3 fimbriae in the formation of biofilm in *K. pneumoniae*[82].

Curiously, in healthy individuals the production of fimbriae by *K. pneumoniae* and its binding to biotic surfaces increases their interaction with immune cells. Thus, the binding of bacteria to phagocytes can lead to phagocytosis and bacterial death[82].

2.3.3. Lipopolysaccharides

The outer membrane composition of the bacteria can be translated into highly important features with respect to their virulent potential. Lipopolysaccharides (LPS) are part of the outer membrane composition and have a significant role in *K. pneumoniae*. Despite its structural diversity among the various species, LPS is usually composed of three fundamental parts: O-antigen, oligosaccharide core and lipid A[82].

Lipid A is the closest structure to the bacterial membrane and is an activator of the host inflammatory response. Some bacteria, such as *K. pneumoniae*, can modify their lipid A to make the inflammatory activity less intense during infection. In fact, the modified lipid A has a lower activity than the original lipid A and is actually effective in increasing the virulence of *K. pneumoniae*. In addition, lipid A has the ability to infer protection against cationic antibacterial molecules[82].

O-antigens are the outermost part of the LPS and there are been identified 9 different O-antigens in *K. pneumoniae*[82]. The majority of human-host-associated infections by *K. pneumoniae* have shown that the most prevalent O serotypes are O1 followed by O2 and O3[88]. O-antigen plays a key role in the virulence of *K. pneumoniae* strains because its function ensures protection against the complement system. Strains with a complete O-antigen are longer in length and resistant to complement-mediated death. Strains with an incomplete or missing O-antigen are susceptible to complement and consequently to erradication[82].
In fact, the role of LPS in *K. pneumoniae* virulence is quite important, as are the genes encoding the proper production of these lipopolysaccharides. Essentially, two genes are recognized, *uge* and *wab*G. The *wab*G gene is found in most, if not all, clinical isolates of *K. pneumoniae* since strains lacking this gene are unable to form the oligosaccharide core of the LPS and to retain capsular antigens. The *uge* gene is also present in most *K. pneumoniae* isolates and its absence leads to the production of LPS with incomplete O-antigen, which indicates a lower survival capacity of the bacteria against complement human system[89].

2.3.4. Siderophores

Iron is one of the most important macronutrients for the development of all microorganisms, but despite being abundant in the environment, it is not in the ideal form to be used by them. In part, iron is unavailable because the presence of oxygen rapidly leads to its oxidation and subsequent formation of insoluble compounds. On the other hand, the availability of iron in nature is much lower than the need for microorganisms, being insufficient for their normal growth and physiological and metabolic processes[90]. Thus, the ability to acquire iron during infection is necessary for the pathogenesis of *K. pneumoniae*, which competes with host cells for the acquisition of this important cofactor[91]. Consequently, *K. pneumoniae* has the ability to produce small iron chelating molecules named siderophores, these iron acquisition systems include the enterobactin, aerobactin, salmochelin and yersiniabactin[92,93].

The role of siderophores in multiplication and development of virulence is described in several pathogens, including *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus*. During the infection, extracellular iron is bound to transferrin proteins, which hinders its access by pathogenic bacteria. Siderophores act competitively with these proteins since they have a high affinity for Fe^{3+} ions, breaking one of the host's immunity barriers[90]. Each *K. pneumoniae* strain can encode more than one siderophore type, which may be a strategy to better colonize the host, given that different siderophores may colonize different tissues and as a precautionary measure in case the host cells neutralize one of the siderophores. In addition, iron affinity is variable between siderophores, enterobactin has the highest affinity and aerobactin has the lowest[93,82].

Enterobactin is expressed in almost all strains of *K. pneumoniae*, being equally present in common strains of *K. pneumoniae* and hv-KP and is therefore considered to be the main iron acquisition system for infections caused by this bacterium[93,82]. Despite being the most common and most affective iron ion siderophore, enterobactin is neutralized by lipocalin-2, an antibacterial-capable molecule released by various host cell types during infection and an activator of inflammatory response. Lipocalin-2 has no bactericidal effects, however it prevents the growth of *K. pneumoniae* as it deprives iron. As a consequence, strains encoding only this type of siderophore have a high difficulty in colonizing the host[82].

Although yersiniabactin is mostly present in hv-KP isolates (about 90 %), together with enterobactin it is overexpressed in clinical isolates of *K. pneumoniae* of the respiratory tract[93], which occurs because unlike enterobactin, yersiniabactin is not inhibited by the action of lipocalin-2, that allows *K. pneumoniae* to develop quite a lot in the lungs during the infection. On the other hand, yersiniabactin is susceptible to the action of host transferrin proteins, so strains expressing only this siderophore are unable to acquire the iron they need and are unable to spread in the lungs. Thus, yersiniabactin-producing strains alone should not be able to infect immunocompetent individuals[82].

Another siderophore expressed in *K. pneumoniae* strains is salmochelin, a glycosylated form of enterobactin. Glycolylation of this molecule allows lipocalin-2 to fail to bind, which in turn prevents neutralization of the siderophore and activation of an inflammatory response from the host. Such as yersiniabactin, salmochelin is mostly present in hv-KP strains, and the presence of this siderophore in 90 % of hv-KP strains associated with pyogenic liver abscess has been reported[93,82].

Aerobactin is a citrate hydroxamate siderophore that is almost exclusively expressed by hv-KP strains. Interestingly, there are data suggesting that hv-KP strains have a greater ability to produce siderophores, especially aerobactin, than nonvirulent strains, which may contribute to virulence and pathogenesis[93]. Furthermore, expression of this siderophore only occurs in strains having a hypercapsule (known virulence factor), although not all hypercapsulated *K. pneumoniae* strains have aerobactin[82].

2.3.5. Biofilm Formation

Biofilm formation has been considered an important factor in *K. pneumoniae* pathogenesis, especially in patients who have undergone medical procedures involving the insertion of devices such as endotracheal tubes or catheters, in particular in the case of catheter-associated urinary-tract infections and ventilator-associated pneumonia[94]. However, *K. pneumoniae* biofilms can also contribute to the colonization of the gastrointestinal tract[93].

Biofilm consists of an aggregate of cells contained in self-produced matrix of polysaccharides, proteins, DNA, lipids and ions[95]. Its formation is a complex process divided into several stages (Figure 4), from the initial attachment of the bacteria to a surface, to the formation of microcolonies, maturation and finally dispersion of cells[93,96].

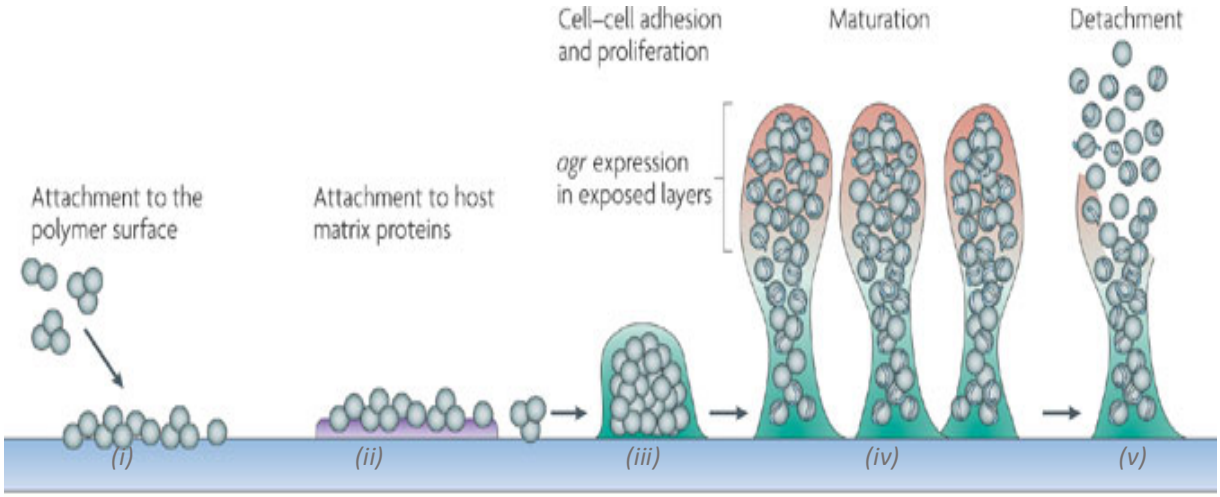


Figure 4- Stages of biofilm development. (i) bacterial cells attach reversibly to the surface, (ii) irreversible attachment, (iii) microcolony formation, (iv) maturation and differentiation of the biofilm, (v) detachment of single motile cells. (Adapted from Otto, 2009)[99].

Biofilm formation actually begins when bacterial cells bind irreversibly to a surface, this is because cells begin to develop and produce extracellular components, such as proteins. As cells replicate, the extracellular components produced make the biofilm increasingly complex, forming microcolonies. Subsequently, the biofilm begins to differentiate and mature, reaching a stage that is characterized by enormous structural complexity[97].

The capacity to form a biofilm provides to bacteria a mechanism to survive and persist in stressful conditions or a changing environment[95]. Biofilms confer protection against, for instance, phagocytosis, UV radiation, shear stress, dehydration, biocides, antibiotics and the host immune response, being increasingly recognized as an important virulence property[98].

Cell growth conditions within the biofilm change, as nutrient availability is lower, as is oxygen perfusion, especially due to cell density, waste removal, and physical and chemical conditions also change. Under these growing conditions, there is a high genetic expression that can justify the complexity of the development process of a biofilm. Changes in gene expression that occur during the process of bacterial biofilm formation imply that there is a phenotypic diversity that is variable at each stage. This phenomenon is especially important as regards the resistance to antibacterial agents of mature biofilms[99]. Normally the cells that form the biofilm surface layer are metabolically active and susceptible to the action of antibiotics,

while the middle and lower zones of the biofilm consist of cells that are normally in a dormant state, and for this reason, even if antibiotics strike them, they have no activity against them[100].

In addition, transcriptional changes occur in bacterial cells to activate their cell-to-cell communication and via quorum sensing to respond to the stimulus that protects the cells against external agents such as antibiotics[101]. Quorum-sensing is a communication system between cells used by bacteria inserted in a biofilm to communicate with each other and regulate gene expression according to the conditions to which they are subjected. The quorum-sensing signal corresponds to small molecules, called autoinducers, that are released by bacteria that bind to intracellular receptors, activating or suppressing gene expression[100]. In this way, biofilm provides to bacteria a mechanism to survive and persist in stressful conditions or a changing environment[95].

A study has demonstrated that about 40 % of *K. pneumoniae* isolates were able to produce biofilm, in particular isolates from urine samples of catheterized patients[102]. More recently, a high rate of *K. pneumoniae* strains from endotracheal tubes of patients affected by ventilator-associated pneumonia were also reported to be able to form an *in vitro* biofilm[102].

Biofilm formation is more observed among isolates from young adults than isolates from seniors. This difference could possibly be explained by the fact that patients under 40 years old have a stronger immune system, forcing the bacteria to create conditions for its survival against host immunity[3].

Notwithstanding, other important virulence factors are involved on the formation of biofilms by *K. pneumoniae* such type 1 and 3 fimbriae and the capsular polysaccharides, discussed in the preceding sections. Type 3 fimbriae allow bacteria to adhere either on biotic or abiotic surfaces. The growth on abiotic surfaces is possible by the presence of *Mrk*A type 3 fimbrial protein, and the on human extracellular matrix (HECM) requires the presence of type 3 fimbrial adhesin (*Mrk*D)[103]. In addition to their importance in the initial steps of biofilm formation, type 1 and type 3 fimbriae seem to be important during biofilm maturation. Nevertheless, *K. pneumoniae* can produced other types of fimbriae with a role on biofilm formation[100].

It has been proved that the capsule and lipopolysaccharides in *K. pneumoniae* contribute to the structure of biofilm communities being responsible for a proper initial coverage of substrate and construction of mature biofilm architecture and the initial adhesion on abiotic surfaces, respectively [102,104].

2.3.6. Clonal diversity

The ability of microbial pathogens to modiffy their characteristics depending on the environment and conditions that surrounds them has allowed a microbial evolution and *K. pneumoniae* was not an exception[105]. Clonal diversification into well-adapted phenotypes to hostile environments, such as human

host, is a sophisticated mechanism used by bacteria to resist against antibiotics and human immune defenses[106,107]. One of the manifestations of phenotypic changes in response to an exterior stimulus, is the appearance of colony morphology variation. Phenotypic diversification has been observed in a variety of bacteria, such as *Burkholderia cepacian, Streptococcus pneumonia*, *P. aeruginosa*, *S. aureus*, *Staphylococcus epidermidis, Staphylococcus Capitis, Enterococcus faecalis*, *Salmonella* serovars, *Vibrio cholerae*, *Brucella melitensis*, *E. coli*, *Lactobacillus acidophilus*, *Serratia marcescens* and *Neisseria gonorrhoeae*[107]. Whit this, it would be interesting to observe the morphology of different resistant strains of *K. pneumoniae.*

One of the most problematic phenotypes is the small colony variants (SCV), that are related to recurrent chronic and recurrent infections, as cystic fibrosis[108], device related infection as bone and join infections and osteomyelitis[109]. SCV display augmented resistance to several classes of antibiotics[106] and they are excellent biofilm formers[107], contributing to, for instance, *P. aeruginosa* and *S. aureus* persistence in human host[106,107,110]. Clonal diversification of *K. pneumoniae* has been poorly explored. Recently, Silva *et al.* reported the role of SCV in *K. pneumoniae* resistance to colistin[111]. Since bacteria have the adaptive capacity to group in biofilms in way to protect themselves, the clonal diversification could be related to this adaptation. In this study they verified the appearance of small SCV in the *K. pneumoniae* biofilms, which proved to be resistant to colistin without altering its viability, confirming the altered behaviour of the bacteria through the morphology of their colonies. Therefore, it seemed relevant to invest more efforts in the study of clonal diversification to better understand *K. pneumoniae* adaptation and evolution, with the goal of predict their behaviour, at the level of pathogenesis and resistance to antimicrobial agents in order to aid in its diagnostic recognition.

2.3.7. Hypermucoviscosity

Hypermucoviscosity is one of the most important factors associated to the virulence of *K. pneumoniae* and it was described for the first time in the mid-1980s and 1990s associated to a unique clinical syndrome of community-acquired *K. pneumoniae* infections[112].

This *K. pneumoniae* variant showed unique clinical features when compared to the wild-type *K. pneumoniae* and even other pathogens with potential to cause nosocomial infections. First, it had a strong ability to cause serious infections on immunocompetent, young and healthy patients[113], while wild-type *K. pneumoniae* is opportunistic. Second, unlike other Gram-negative bacilli, this *K. pneumoniae* variant had some propensity to causing metastatic infections[114], which are characterized by the expansion of the bacterium to other sites of infection. In *K. pneumoniae* liver abscesses, the rate of metastatic infection ranges from 3,5 % to 20 %[115]. Moreover, this *K. pneumoniae* variant exhibited different bacterial phenotypic

features. Its colony appearance when grown on agar medium was highly viscous (not equal to being mucoid) and thus designated as hypermucoviscous. In fact, this phenotypic feature has been used in the laboratory as a standard test to distinguish hypervirulent *K. pneumoniae* (hv-KP) from wild-type *K. pneumoniae*[116,117].

The expression of the hypermucoviscous phenotype is mediated for *Rmp*A/ *Rmp*A2 (regulator of the mucoid phenotype) which regulates capsule production, and *Mag*A, associated with hypermucoviscous phenotype[9]. However, genes reported to encode *Rmp*A/ *Rmp*A2 are also present in wild-type *K. pneumoniae* strains[6]. Thus, it is necessary to develop studies that allow a better understanding of the role of *Rmp*A in the mediation of virulence in strains of *K. pneumoniae*. In addition to the *Mag*A gene being a mediator to the production of this phenotype, some studies have demonstrated that *Mag*A also encodes a polysaccharide polymerase enzyme that is specific to capsular serotype K1 and responsible for the formation of capsular structure[118].

Capsular polysaccharides serotypes K1 and K2 are the most mentioned in association with hv-KP[7]. These observations could indicate that the fact of K1 and K2 serotypes being present, mostly in hv-KP, confer more virulence, compared to strains from other serotypes. However, K1 and K2 serotypes are not exclusive from hv-KP strains, some wild-type *K. pneumoniae* strains also acquire these capsular polysaccharides serotypes. In fact, wild-type *K. pneumoniae* strains with K1 and K2 serotypes are not significantly less virulent than K1 and K2 hv-KP strains[119]. These evidences seem to indicate that the presence of serotype K1 or K2 in isolates of *K. pneumoniae* does not give them a hypervirulent phenotype, but that a higher expression of capsular material (hypermucoviscous phenotype) in combination with a higher expression of siderophores, that mediate iron acquisition, enhancing the growth and survival in the host, are responsible for the increase of the virulence level observed, conferring a status as hypervirulent phenotype (hv-KP)[78,6].

The ability to produce biofilms is also recognized as an important virulence property of hv-KP strains. Wu *et al.* demonstrated that hv-KP strains easily produced more biofilm than wild-type *K. pneumoniae* isolates, supporting the hypothesis that biofilm formation may be a contributing factor for its increased virulence although the mechanisms are not be fully known[103].

The first infections associated with hv-KP strains appeared in the Asian Pacific Rim, however the problem of their extreme virulence led to the emergence of these strains around the world, and cases have been reported in America, Europe and Africa. Although hv-KP is highly associated with virulent strains of *K. pneumoniae* and has been shown to be susceptible to most antibiotics, which makes it even more different from wild-type *K. pneumoniae*, reports of MDR and ESBL-producers strains of hv-KP are recently emerging in China[116].

2.4. Dissemination of *Klebsiella pneumoniae* antibiotic resistance

The spread of antibiotic-resistant pathogens has become a serious problem worldwide as it makes it difficult to treat infectious diseases, and this is even more alarming due to the increase in resistant bacteria capable of causing both nosocomial infections and community-acquired infections. Nowadays, are particularly worrying ESBLs and carbapenemases-producing bacteria due to the astronomical increase of the number of reported infections. The production of ESBLs and carbapenemases is a mechanism of antibiotic resistance already verified in several species, besides *K. pneumoniae,* these enzymes are found in *E. coli*[120], *P. aeruginosa*[121], *P. mirabilis*[122], *Salmonella*[123], *Shigella*[124], *V. cholerae*[125], *S. aureus and A. baumannii*[16].

Initially, the production of ESBLs was detected in species of the Enterobacteriaceae family. The first ESBL was reported in an *E. coli* strain, however its production was rapidly detected in *K. pneumoniae* and other species of the same family[126]. The use of antibiotics in the fight against bacterial infections increased the selective pressure on the bacteria which promoted the survival of the β-lactamases producing strains.

Most infections caused by pathogenic bacteria are of polymicrobial origin, and these are organized in a complex where the interactions between them dictate the characteristics of the population, such that bacteria that are unable to form a biofilm, can, by synergism, form a biofilm with other bacterial species[95]. The interaction between the members within polymicrobial consortia, such as competition, commensalism, mutualism and parasitism, are the key behind the improved survival of microorganisms. The formation of biofilm is an essential factor in the process of colonization and persistence of infection, which guarantees protection to the population of which it is constituted. The growing conditions of a biofilm-based population allow for a stronger response against the host immune system and trigger increased resistance to antimicrobial agents. Increased antimicrobial resistance in biofilms is due in part to the ease of communication between cells and their proximity that facilitates horizontal gene transfer[127]. Therefore, biofilms provide the ideal conditions for the exchange of genetic material and for this reason, constitute a huge scourge of public health because they privilege the spread of resistance among other species and throughout the world.

During infection in the host, *K. pneumoniae* is not isolated but in contact with other species[128]. Their ability to grow in contact with *P. aeruginosa* has been demonstrated and it appears that the presence of both species does not interfere with their individual growth[129]. Furthermore, *K. pneumoniae* and *P. aeruginosa* are often found together in chronic biofilm-mediated infections, notably urinary tract, respiratory tract and burn wounds[95,130]. In addition to *P. aeruginosa*, there are other microorganisms which forma mixed-species biofilm with *K. pneumoniae*, such *P. mirabilis*, on the presence of *E. coli*, *Morganella morganii* or E*nterobacter cloacae*, *Candida albicans* and *Streptococcus*[130].

The formation of mixed biofilms, in which *K. pneumoniae* is present, is of high clinical interest as they contribute to increased resistance to various antibiotics and are involved in chronic infections. The associated biofilm infections are increasingly worrisome due to their persistence and resistance.

2.5. Management of *Klebsiella pneumoniae* infections

The current situation at hospital level in order to control nosocomial infections caused by pathogens is indeed disturbing. The rise of infections caused by pathogenic microorganisms is increasingly frequent and the problem is even more seriously when the infection is caused by microorganisms belonging to the ESKAPE group. In these cases, the infection control is extremely complicated and the treatment options very scarce. *K. pneumoniae* is included in this group of pathogens, and the frequency of clinical isolates with multi-resistant phenotypes is alarming. In addition to the ESBLs and CP-KP producing *K. pneumoniae* strains capable of hydrolyse most of the available antibiotics such as penicillins, cephalosporins, monobactams, carbapenems and β-lactamase inhibitors[3,5], MDR and XDR strains now appear, which contain several resistance genes making the choose for the appropriate antibiotic almost impossible.

Infections associated with ESBL-producing *K. pneumoniae* strains are related to some risk factors that include the use of antibiotics of the same class in the treatment of disease in the past, prolonged hospitalization in hospitals and clinics, especially in ICUs and mechanically ventilated sites[131]. The predisposition of the host for the contraction of CR-KP infections is related to the previous use of antibiotics for the treatment of other infections, renal dysfunction, older age, surgical procedures and admission on ICU[132].

Due to the difficulties encountered in the treatment of infections caused by resistant *K. pneumoniae* strains, and the inefficiency of antibiotics against these cases, a new strategy for combining antimicrobial agents has been used today. This strategy consists of administering specific combinations of different types of antibiotics, including polymyxins, carbapenems, gentamicin, fosfomycin, rifampicin and others. Among the several studies reviewed, the most efficient and administered combination of agents for treatment of the infection caused by CR-KP is tigecycline-colistin, carbapenem-colistin and tigecycline-gentamicin. In fact, although the CP-KP infection mortality rate remains high, the combination of antibiotics is more efficient than monotherapy[5,60,133].

Polymyxins, as colistin, are cationic cyclic polypeptides that are linked to a fatty acid chain, their mechanism of action involves an initial interaction of the cationic lipopeptide with lipid A of lipopolysaccharide (LPS) in the exterior membrane, moving divalent cations (Ca^{2+} and Mg^{2+}) from the negatively charged phosphate groups of lipid A, followed by uptake across the exterior membrane. The

hydrophobic interaction between the fatty acid chain of polymyxins and lipid A also plays an important role in the first step of polymyxin action[60]. As most of the drugs, polymyxins have some adverse effects, causing nephrotoxicity and neurotoxicity. Nephrotoxicity appears in 50 % of patients and specially on those critically ill[60]. Adverse effects are usually reversible upon the discontinuation of the antibiotics. Although polymyxins maintain effective against most CRE, some reports of colistin-resistant carbapenemase-producer *K. pneumoniae* strains have emerged. This resistance seems to be related with alterations in regulatory system and modifications of lipopolysaccharide[60].

Tigecycline is a derivate of minocycline design to circumvent efflux-mediated resistance mechanisms. This antibiotic can be use in both infections with Gram-positive and Gram-negative bacteria, including CRE. This antibiotic kinetics results in low concentrations in blood, epithelial fluid of the longs and urinary tract[60]. Consequently, it is not very used for the treatment of patients with bacteraemia, pneumonia and UTI. However, nonsusceptibility to tigecycline is become common in carbapenemase-producer *K. pneumoniae*, occurring in patients who have been treated with this agent before[60]. Although its use is effective against CRE *in vivo*, its use is carried out in conjunction with other agents in order to overcome their individual limitations.

Despite *in vitro* studies demonstrated that gentamicin exert a bactericidal activity against CR-KP, its bactericidal power does not extend to all strains and, therefore, in clinical practice, gentamicin is commonly used in combination with colistin, a carbapenem or tigecycline[60].

The combination of antibiotics in the treatment of infections caused by resistant *K. pneumoniae* strains has been the most successful strategy in recent times, especially the combination of different classes, which allows the action of antibiotics with different mechanisms of action. However, there is still a long way to go especially with the emergence of *K. pneumoniae* MDR and XDR strains.

Chapter 3. Materials and Methods

3.1. Bacterial strains and cultures conditions

The present study was conducted using 26 strains of *K. pneumoniae* including a reference strain ATCC 11296 and 25 clinical isolates. The clinical isolates were obtained from different Portuguese hospitals and some information was previously obtained from the clinical records (Table 1). All bacterial strains were routinely cultured on Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) medium at 37 °C. Bacteria were preserved in cryovials (Nalgene) at – 80 ± 2 °C. Prior to each experiment, bacterial cells were grown on TSA plates for 24 h at 37 °C.

3.2. Antimicrobial susceptibility test of *Klebsiella pneumoniae* isolates

The antimicrobial susceptibility was performed for those *K. pneumoniae* strains there were no information (Table 1). Thus, the disk diffusion method was used because it is a cheaper and simple method that does not require much equipment and material providing fast and easily interpreted results[134]. Disk diffusion susceptibility testing was performed in accordance with the recommendations of NCCLS document M100-522[135]. First, bacteria were grown overnight on TSB at 37 °C and 120 rpm and further collected by centrifugation at 9000 *g* for 5 min at room temperature, washed and suspended in sterile water. Cell concentration was adjusted to $\mathbf{1 \times 10^8}$ CFU/mL and spread on Mueller-Hinton agar (Sigma-Aldrich) plates. After that, commercially prepared antibiotic discs were placed on the agar surface and the plates were incubated for 18 to 21 h at 37 °C. The antimicrobial susceptibility was determined for the following antibiotics: 30 μg cefotaxime (CTX), 30 μg ceftriaxone (CRO), 30 μg ceftazidime (CAZ), 30 μg cefepime (FEP), 30 μg tetracycline (TE), 100 μg piperacillin (PRL), 10 μg gentamicin (CN), 5 μg levofloxacin (LEV), 30 μg aztreonam (ATM) and 10 μg imipenem (IMI). All discs were purchased from Liofilchem. The interpretation of the results was also made according CLSI guidelines and *K. pneumoniae* strains were categorized as susceptible (S), intermediate (I), dose-dependent susceptible (SSD) and resistant (R). The interpretative criterion SSD has recently emerged due to changes in the specific dose regimen and it only affects the cefepime breakpoint. This new criterion replaces the intermediate classification and proposes new more effective dosing regimens. Isolates with a susceptibility result of SSD category, requires higher or more frequent doses, which results in greater exposure to cefepime[135].

Table 1. Information concerning the strains used throughout this study regarding their isolation site and susceptibility profile. ND – no data; • Present; "S" susceptible; "I" intermediate; "R" resistant.

Strain	Source	Resistance genes				Antimicrobial agents									
		ESBLs			Carbapenemases										
		SHV	TEM	CTX	KPC	Imipenem	Cefotaxime	Ceftazidime	Cefepime	Ceftriaxone	Piperacillin	Aztreonam	Levofloxacin	Gentamicin	Tetracycline
Isolate 1	Expectoration	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isolate 2	Ocular extraction	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isolate 3	Spot urine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isolate 4	Expectoration	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isolate 5	Urine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isolate 6	Urine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isolate 7	Urine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isolate 8	Expectoration	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isolate 9	Urine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isolate 57	Expectoration	•			•	ND	R	R	I	ND	R	ND	ND	S	ND
Isolate 63	Skin exudate	•	•	•		ND	R	R	I	ND	R	ND	ND	R	ND
Isolate 64	Pus	•	•	•		ND	R	R	S	ND	R	ND	ND	S	ND
Isolate 66	Spot urine		•		•	ND	R	R	ND	ND	R	ND	R	R	ND
Isolate 67	Expectoration	•	•	•		ND	R	R	ND	ND	R	ND	R	R	ND
Isolate 68	Expectoration	•	•		•	ND	R	R	ND	ND	R	ND	S	R	ND
Isolate 69	Spot urine	•				ND	R	R	ND	ND	R	ND	S	S	ND
Isolate 70	Blood	•		•		ND	R	R	ND	ND	R	ND	R	R	ND
Isolate 71	Bronchial aspirate	•		•		ND	R	R	ND	ND	R	ND	R	S	ND
Isolate 89	Blood	•	•	•		ND	R	R	R	ND	R	ND	ND	R	ND
Isolate 90	Expectoration	•	•	•		ND	R	R	R	ND	R	ND	ND	R	ND
Isolate 91	Bronchoalveolar lavage	•	•	•		ND	R	R	R	ND	R	ND	ND	S	ND
Isolate 92	Pus	•				ND	R	R	S	ND	R	ND	ND	S	ND
Isolate 93	Blood	•	•	•		ND	R	R	I	ND	R	ND	ND	R	ND
Isolate 94	Skin exudate	•	•			ND	R	R	I	ND	I	ND	ND	R	ND
Isolate 95	Expectoration	•	•	•		ND	R	R	I	ND	R	ND	ND	R	ND

3.3. Phenotypic detection of Extended-spectrum β-lactamases

K. pneumoniae strains with altered susceptibility to cefotaxime, ceftazidime, imipenem and piperacillin were used to investigate the production of ESBL. The phenotypic detection of ESBL production was performed by Modified Double Disc Sinergy Test (MDDST). The MDDST methodology consists on the interaction between drugs for the detection of ESBL-producing strains. A disc with a β-lactamase inhibitor was placed in the center of the plate and cephalosporin discs were placed around it (20 mm apart).

Similar to disc diffusion test and following NCCLS criteria[135], 1×10^8 CFU/mL of overnight cultures were spread on Mueller-Hinton agar plates. Discs containing 30 μg amoxicillin-clavulanate, were placed on the center of the plates and surrounded by four cephalosporins discs placed 20 mm apart, from each other. Third generation cephalosporins cefotaxime, ceftazidime and ceftriaxone, and fourth generation cefepime were used. Further, agar plates were incubated at 37 °C for 18 - 21 h. The synergism between a cephalosporin and clavulanic acid is detected by a distorted inhibition halo or an increase of the inhibition zone of inhibition toward the clavulanic acid disk, confirming the ESBL production.

3.4. Characterization of the virulence factors expression of *Klebsiella pneumoniae* isolates

3.4.1. Phenotypic detection of hypermucoviscosity

Hypermucoviscosity was qualitatively measured by a string test[6]. Overnight cultures of approximately 1×10^3 CFU/mL were spread onto Columbia blood agar plates (Liofilchem) with 5 % horse blood (v/v) and allowed to grow overnight at 37 °C. A loop was used to stretch one colony and the formation of a mucoviscous string superior to 5 mm was considered indicative of a hypermucoviscosity phenotype.

3.4.2 Blood Hemolysis

Hemolytic activity of *K. pneumoniae* was evaluated by streaking approximately 1×10^3 CFU/mL of *K. pneumoniae* isolates on Columbia blood agar plates (Liofilchem) with 5 % horse blood (v/v)[136]. Hemolysin negative production was defined when no clearing zones were observed around the isolated colonies, after 24 h of growth at 37 °C.

3.4.3. Observation and classification of colony morphology

The colony morphology of the *K. pneumoniae* resistance isolates, was determined as previously described[106]. Serial bacterial dilutions of overnight cultures washed twice with sterile water were plated in TSA at 37 °C. After 24 and 45 h of incubation, the colonies formed were observed, photographed using

magnifying glass (Olympus SZ- CTV) and the images recorded with a CCD camera (AVC, D5CE, Sony, Tokyo, Japan). The classification of colony morphology was carried out using nine parameters: colony form, margin, texture, color, consistency, size, type of surface, elevation and opacity, according to Table 3.1 of the appendix[137]. A colony morphotype was determined when a colony differ in, at least, one morphological trait. All experiments were performed 3 times.

3.4.4. Phenotypic detection of biofilm formation

The phenotypic detection of biofilm formation was first qualitatively evaluated as previous described[138]. Overnight cultures of approximately 1×10^3 CFU/mL were spread onto Brain heart infusion agar supplemented with 5 % (w/v) sucrose and 0,08 % (w/v) Congo red. Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121 °C for 15 min. Then, it was added to Brain heart infusion agar with sucrose. Plates were incubated at 37 °C for 24 to 48 h. Black colonies with a dry crystalline consistency indicated biofilm production and non-biofilm producers usually remained red.

3.4.5. Biofilm formation and biomass quantification

Biofilms were developed as previously described[3]. *K. pneumoniae* strains were grown overnight on TSB at 37 °C under agitation (120 rpm). Cell suspensions were washed twice with sterile water and diluted in TSB in order to obtain the final concentration of 1×10^7 CFU/mL. Posteriorly, 200 µL of the bacterial suspensions were transferred to a 96-well polystyrene microtiter plate (Orange Scientific) where biofilms were developed aerobically on a horizontal shaker (120 rpm) at 37 °C for 24 h.

Biofilm formation was quantified by crystal violet (CV) method[3]. After biofilm formation, the liquid content of the plates was discarded, and plates were washed twice with sterile water in order to withdraw weakly attached cells. Afterwards, 200 µL *per* well of pure methanol was added and plates were allowed to stand for 15 min in order to fix the biofilm. After methanol discharge, plates were left to dry at room temperature. The fixed biofilms were then stained with 200 µL of 1 % (v/v) CV, for approximately 5 min. The CV bound to the adherent biofilms was solubilized with 200 µL of 33 % (v/v) acetic acid. The OD of the obtained solution was measured at 570 nm using a microtiter plate reader (Bio-Rek Synergy HT, Izasa).

3.4.6. Composition of the extracellular polymeric matrix

Part of the biofilm polymer matrix composition was inferred using two different enzymatic treatment on biofilm formation, as described previously[139]. For this experiment, *K. pneumoniae* clinical isolates 66, 70, 90 and 94 were used. Biofilms were developed as describe above (Section 3.4.5) and after 24 h, the wells

were washed twice with sterile distilled water to remove non-adherent bacteria and filled with 100 µg/mL DNase I (Grisp, Porto, Portugal) in the reaction buffer advised by enzyme brand or with 100 µg/mL Proteinase K in acetate buffer 0,1 M pH 5.0. The treatments were allowed to act at 37 °C for 4 h. Biofilms incubated with reaction buffer or acetate buffer were used as controls. After the treatments, biofilms formed in the 96-well polystyrene microtiter plates were washed with sterile distilled water and biofilms biomass was quantified by CV method as earlier describe in the section 2.4.5.

3.5. Dissemination of antibiotic resistance in polymicrobial communities

3.5.1. Mixed biofilm formation

Dual-species biofilms of *K. pneumoniae* strains and *P. aeruginosa* PAO1 were developed as previously described[140]. To form the 24-h old biofilms, overnight bacterial cultures of each specie were diluted in TSB in order to obtain 5×10^6 CFU/mL as final concentration. Posteriorly, 100 µL of each bacterial suspension were transferred to a 96-well polystyrene microtiter plate (Orange Scientific), obtaining a final concentration of 1×10^7CFU/mL, where dual-species biofilms were allowed to form aerobically on a horizontal shaker at 120 rpm and 37 °C for 24 h. A similar procedure was implemented to form 72-h old biofilms. Biofilms were formed in 24-wells microtiter plates (Orange Scientific), bacterial suspensions were diluted in TSB as previously. 1,5 mL of bacterial suspension was introduced in each well, where dual-species biofilms were allowed to form aerobically on a horizontal shaker at 120 rpm and 37 °C for 24 h.

3.5.2. Counting the number of viable cells

The number of viable cells obtained from biofilms was inferred through colony-forming unit (CFU). In the 24-h old biofilms, the liquid content of the plates was discarded, and the wells washed twice with sterile distilled water in order to remove the weakly attached cells. Further, 200 µL of sterile distilled water were added in each well, and biofilms were detached using an ultrasound bath (Sonic model SC-52, UK) for 10 min[140]. The 72-h old biofilms were also washed twice with sterile distilled water in order to remove the weakly attached cells. Further, 1,5 mL of sterile deionized water were added in each well, and instead of using the ultrasound bath, the wells were scraped in order to collect the biofilm biomass. The content of wells was recovered and gently vortexed to homogenize the biofilm-cells suspension for spreading onto solid medium.

3.5.3. Quantification of biofilm biomass

Quantification of the biomass of the 24-h old biofilms was performed through the CV method as described in section 3.4.5.

3.5.4. Dissemination of antibiotic resistance to *P. aeruginosa* PAO1

The spread of resistance between species was evaluated after the formation of mixed biofilms. After 24 h, biofilm content was obtained, and the bacterial suspensions were gently vortexed to homogenize and further diluted. Bacteria was allowed to grow overnight in two separate media, LB medium which served as a growth control for both species, and LB medium supplemented with 4 μg/mL ceftazidime (CAZ) to verify growth of ceftazidime resistant PAO1. Simultaneously, the number of viable cells was counted as described above.

3.5.5. Mixed biofilm resistance to ceftazidime

Dual-species biofilms of *K. pneumoniae* strains and *P. aeruginosa* PAO1 were developed as previously described[140]. To form the 24-hours biofilms, overnight bacterial cultures of each specie were diluted in TSB in order to obtain 5×10^6 CFU/mL as final concentration. Posteriorly, 100 μL of each bacterial suspension were transferred to a 96-well polystyrene microtiter plate (Orange Scientific), obtaining a final concentration of 1×10^7CFU/mL, where dual-species biofilms were allowed to form aerobically on a horizontal shaker at 120 rpm and 37 °C for 24 h. After biofilm formation, the contents of the wells were removed and a solution of 8 mg/L ceftazidime (CAZ) in TSB was added to each well, the biofilm were incubated for a further 24 h to allow antibiotic action.

The number of viable cells and the quantification of 24-h old biofilms biomass were performed as described in the section 3.5.2. and 3.4.5., respectively.

3.6. Statistical analysis

Averages and standard deviations are shown for each graph, derived from three independent assays. All statistical analyses were performed by different statistical tests using Prism Software (GraphPad version 7.0), considering as statistically significant a *p*-value less than 0,05. The association tests performed in sections 4.3.2 and 4.3.3 were performed with the support of the SPSS version 26.

Chapter 4. Results and Discussion

K. pneumoniae have experienced a dramatic increase of resistance in the past decades, possessing numerous mechanisms of antibiotic resistance which have a huge impact on effective treatments[9]. The indiscriminate use of antibiotics has led to the emergence of resistance strains producing ESBLs and, lately, carbapenemases posing new challenges in therapeutics and infection control[9,141]. The *K. pneumoniae* strains are often resistant to several classes of antimicrobial agents beyond β-lactams, including tetracyclines, aminoglycosides and fluoroquinolones[142]. Therefore, the emergence and spread of antibiotic resistance in *K. pneumoniae* becomes a global problem affecting clinicians, drug manufacturing, industries and healthcare agencies.

The biological success of *K. pneumoniae* can be attributed in part to their preference to form biofilms. Current knowledge of ecology and physiology of *K. pneumoniae* biofilms is necessary to understand the disease burden and to take necessary actions to prevent biofilm formation and spread of resistance. Therefore, this study aimed to investigate the ecology and physiology of *K. pneumoniae* biofilms and understand how they provide antibiotic tolerance to bacteria to further use this knowledge to formulate effective antibiotic strategies and plan proper hospital infection control strategies to prevent the spread of *K. pneumoniae*. The relevance of this type of study is progressively more important worldwide due to the emergence of increasingly resistant strains of *K. pneumoniae* and, moreover, hypervirulent strains.
Throughout this study, 25 clinical isolates of *K. pneumoniae* and a reference strain *K. pneumoniae* ATCC 11296 were used.

4.1. Susceptibility profile of *Klebsiella pneumoniae* isolates

Nine of the 25 isolates did not have information about their antimicrobial resistance profile (Table 1), and, thus, an antimicrobial susceptibility testing was performed. The antimicrobial susceptibility testing included 10 different antibiotics belonging to 7 different classes (Table 2) relevant in the treatment of *K. pneumoniae* infections. Four different cephalosporin antibiotics were tested as they belong to the group of β-lactam agents used to treat *K. pneumoniae* infections. The number of strains resistant to these antibiotics is increasing globally and third generation cephalosporins are no longer an option, so third and fourth generation cephalosporins were used.

The determination of antimicrobial susceptibility was performed according to the clinical breakpoints of CLSI and bacteria classified as susceptible (S), intermediate (I), dose-dependent susceptible (SDD) and resistant (R)[135].

Table 2. Antimicrobial susceptibility test of 9 clinical isolates of *K. pneumoniae* and *K. pneumoniae* ATCC 11296 used as a control strain. " S" stands for susceptible strain, " R" stands for resistant strain, " I" for intermediate and " SDD" stands for susceptible-dose dependent according to CLSI breakpoints. The final result was achieved after 2 of 3 equal independent repeats.

Antibiotic class	**Carbapenem**	**Cephalosporins**				**Penicillin**	**Monobactam**	**Fluoroquinolone**	**Aminoglycoside**	**Tetracycline**
Antibiotic	Imipenem	Ceftazidime	Cefepime	Cefotaxime	Ceftriaxone	Piperacillin	Aztreonam	Levofloxacin	Gentamicin	Tetracycline
K. pneumoniae ATCC 11296	S	S	S	S	S	S	S	S	S	S
Isolate 1	S	R	SDD	R	R	R	I	S	R	S
Isolate 2	S	S	S	S	S	S	S	S	S	S
Isolate 3	S	S	S	S	S	S	S	S	S	S
Isolate 4	S	I	R	R	R	R	R	R	R	R
Isolate 5	S	S	S	S	S	R	S	S	S	R
Isolate 6	S	S	S	S	S	S	S	S	S	S
Isolate 7	S	S	S	S	S	S	S	S	S	S
Isolate 8	S	R	R	R	R	R	R	R	R	R
Isolate 9	S	R	R	R	R	R	R	R	R	S

K. pneumoniae ATCC 11296 was used as a control strain for the method and it showed to be susceptible to all antibiotics tested, as expected[143]. Five clinical isolates (isolates 1, 4, 5, 8 and 9) demonstrated resistance to several antibiotic classes and 4 (isolates 2, 3, 6 and 7) were susceptible to all antibiotics tested (Table 2). The isolates 1, 4, 5, 8 and 9 exhibited widely different susceptibility profiles, but they were all resistant to piperacillin. These results reinforced the problem associated with antibiotic resistance and recall its origin, since the excessive use of penicillin-class antibiotics led to the emergence of antibiotic resistance mechanisms[39]. Interestingly, in this study, all 9 clinical isolates of *K. pneumoniae* resistant to other classes of antibiotics were resistant to penicillins.

It should be noted that the isolates 1, 4, 8 and 9 were resistant to relevant class of antibiotics including cephalosporins. These results can reflect the excessive use of penicillin and cephalosporin classes that culminate in the emergence of resistant bacteria. Moreover, co-resistance to other antibiotic classes such as fluoroquinolones, aminoglycosides and tetracyclines by *K. pneumoniae* strains is also of concern, as strains become resistant not only to β-lactams but also to other antibiotic classes, making treatment of infections more difficult[14]. As it is well known, over time bacteria have developed intrinsic resistance to various antibiotics, either by co-existing in the same environment or due to overuse of antibiotics. The selective pressure caused by the uncontrolled use of antimicrobial agents has led to the acquisition of antibiotic resistance genes that has been enriching *K. pneumoniae* resistome to the present day of concern, which is multi-resistance[2].

Nevertheless, all 9 isolates were susceptible to imipenem, a carbapenem antibiotic, suggesting that none of the isolates were a carbapenemase producer, and thus there is still a last antibiotic resort to eradicate these bacteria.

After this analysis, the total sample of 25 *K. pneumoniae* isolates were composed by 76 % (19) isolates resistant to ceftazidime and cefotaxime, 16 % (4) to ceftriaxone, 28 % (7) to cefepime, 20 % (5) to piperacillin, 12 % (3) to aztreonam and tetracycline, 24 % (6) to levofloxacin and 56 % (14) to gentamicin.

The impressive number of ESBLs-associated infections described in literature and their poor clinical outcomes[41] prompted the screening of ESBL producers among the *K. pneumoniae* resistant strains identified in this study. β-lactamase production is perhaps the most important mechanism of resistance to penicillins and cephalosporins and the monobactam aztreonam[126]. Therefore, the phenotypic detection of ESBL production was only performed for the previous isolates with altered susceptibility to cephalosporins, including ceftazidime, cefepime, cefotaxime and ceftriaxone, and penicillin, such as to piperacillin.

The phenotypic detection of ESBLs in *K. pneumoniae* 1, 4, 5, 8 and 9 isolates was accomplished using the modified double disc synergy test (MDDST) as described in section 3.3. This test employs a β-

lactamase inhibitor, in this study the amoxicillin-clavulanate, in combination with third generation cephalosporins such as ceftazidime, cefotaxime and ceftriaxone. Since ESBL phenotypes have become increasingly complex and currently they are able to hydrolyze fourth generation cephalosporins[144], a fourth-generation cephalosporin, cefepime, was included in this test.

K. pneumoniae ATCC 11296 was used in this study as a negative control since it is a non ESBL-producing strain[143] and, so, it allowed to infer about the quality control of these assays. Among the analyzed isolates, 3 isolates (1, 4 and 9) were ESBL producers due the synergism between the β-lactamase inhibitor and the cephalosporins (Figure 5 B, C and F, respectively). In ESBL production testing of isolates 1 and 4 was observed synergisms between the β-lactamase inhibitor and the cefepime, ceftazidime, cefotaxime and ceftriaxone discs. In the isolate 9, synergism between the β-lactamase inhibitor and the cefepime, ceftazidime and cefotaxime disks was observed. It must be noted the ability of ESBL produced by isolates 1, 4 and 9 to hydrolyze cefepime, a fourth-generation cephalosporin. Isolates 5 and 8 were non-ESBL producers, because no synergism between antibiotics and the β-lactamase inhibitor was observed.

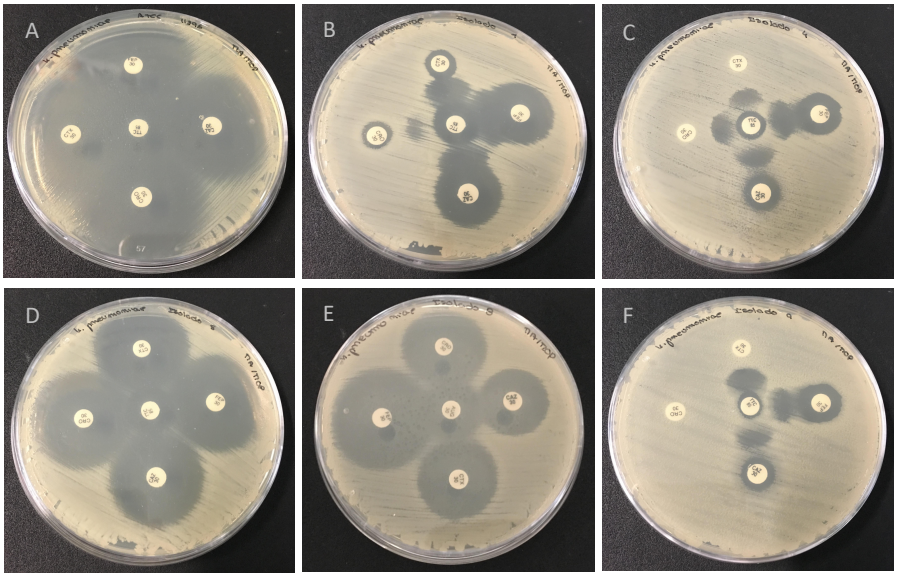


Figure 5- Phenotypic detection of ESBL production by *K. pneumoniae* clinical isolates using modified double disc synergism test. A) Reference strain *K. pneumoniae* ATCC 11296; B) Isolate 1; C) Isolate 4; D) Isolate 5; E) Isolate 8; F) Isolate 9. The final result was achieved after 3 equal independent repeats. (TTC-Ticarcillin-clavulanate, AUG-Amoxicillin-clavulanate, FEP-Cefepime, CTX-Cefotaxime, CRO-Ceftriaxone, CAZ-Ceftazidime).

The problematic associated with ESBL-producing strains is amplified when they are able to infer resistance against other groups of antimicrobial agents other than β-lactams such as aminoglycosides, trimethoprim, sulfonamides, tetracyclines, chloramphenicol and even fluoroquinolones. The susceptibility profile traced for isolates 1, 4 and 9 reflected precisely this scourge. Although clinical isolates produce ESBLs, isolate 1 was also resistant to aminoglycosides, isolate 4 to aminoglycosides, tetracyclines and

fluoroquinolones and isolate 9 to aminoglycosides and fluoroquinolones (Table 2). This broad resistance against various classes of antibiotics is explained by the fact that ESBLs encoding genes are located in large plasmids that harbor resistance genes to other antimicrobial agents, a frequent feature in *K. pneumoniae* isolates[126].

Taking together the results, 76 % (19) of the total sample of 25 *K. pneumoniae* clinical isolates were producers of ESBLs, and 16 % (3) of them were carbapenemases-producers (Table 1). These results reflected the reality of antimicrobial resistance worldwide. The spread of resistance and its prevalence in clinical isolates is even were frightening in cases such as India, where the prevalence rates of clinical isolates producing ESBLs reach almost 70 %[145]. In Portugal, *K. pneumoniae* exhibits an exponential increase in resistance to various classes of antibiotics, especially β-lactams. In just 4 years the percentage of third generation cephalosporin resistant isolates increased by 4 % and the most significant increase was in relation to carbapenems with an increase of 6,8 %[145].

4.2. Characterization of the virulence potential of *Klebsiella pneumoniae* isolates

Pathogenic bacteria such as *K. pneumoniae* have the ability to respond to environmental changes in their local environment in order to make their colonization, growth and even evasion to the host cells more efficient[73]. Virulent *K. pneumoniae* strains are increasingly common throughout the world, and represent a high risk due to their characteristics, being therefore of great importance the study of their pathogenicity factors. Throughout this section some of the major virulence factors associated with the development of *K. pneumoniae* infections were investigated, including hypermucoviscosity, hemolysin production, clonal diversity and biofilm formation. The expression of this virulence factors was performed for those isolates that produced ESBLs and carbapenems. Therefore, the sample included 19 resistant clinical isolates, being 16 ESBLs- and 3 carbapenemases producers, ATCC 11296 and 2 susceptible isolates. In total, 22 strains were used in the following analysis.

4.2.1. Hypermucoviscosity and blood hemolysis

Hypermucoviscosity is one of the most important factors associated to the virulence of *K. pneumoniae*. Hypervirulent *K. pneumoniae* (hv-KP) strains are designated due to their colony appearance that exhibits increased viscosity[6]. Although the hypermucoviscous phenotype is associated with the hypervirulent strains of K. pneumoniae, in the literature it remains uncertain how these characteristics are related[6].

None of the *K. pneumoniae* strains tested, including clinical isolates non- and ESBL and carbapenemases producers, exhibited a hypermucoviscous phenotype. Therefore, the results seemed to indicate that there was no association between the resistance profile of clinical isolates of *K. pneumoniae* and the hypermucoviscous phenotype.

Hemolysins are exotoxins released by bacteria that lead to lyse of the host erythrocytes in order to acquire nutrients[146]. For this reason the production of hemolysins by bacteria is recognized as an important virulence determinant for *K. pneumoniae* pathogenic potential[146].

The results revealed that none of the isolates was able to induce the rupture of the erythrocytes present in the medium, including *K. pneumoniae* ATCC 11296, suggesting that *K. pneumoniae* isolates were not hemolysin-producers. There were no differences among the clinical isolates with distinct resistance profiles, either among ESBLs- and carbapenemases-producing isolates. The absence of hemolytic activity of clinical isolates of *K. pneumoniae* has been described in the literature, regardless of the origin of the isolates or their resistance profile[147].

4.2.2. Clonal Diversity

Clonal diversity has been observed in several bacterial species, however, there is limited information about this mechanism in *K. pneumoniae*. Clonal diversity is frequently associated with antibiotic resistance[148] and thus this feature was investigated in this study.

The study of clonal diversification was based on the number and morphology exhibited by the colonies formed on solid media[106]. Therefore, it is important to ensure that colony traits are well-defined for all strains to accurately compare strains with different resistant profiles. Before the investigation of clonal diversity of the *K. pneumoniae* strains, there was a need to determine the optimal colony growth time correspondent to unaltered colony traits. For that, *K. pneumoniae* planktonic cells were spread onto solid media and colonies observed after 24 and 45 h of growth. This analysis allowed to establish the morphological evolution of colony characteristics of each clinical isolate over time and determine the optimal growth time of colonies to compare them between each other. The identification and characterization of colony morphotypes were performed as previously described[149] and detailed in the Appendix (Table 3.1).

Colony morphotype observed after 24 hours of growth	Possible colony morphotypes evolved from the 24h-old colony morphotype

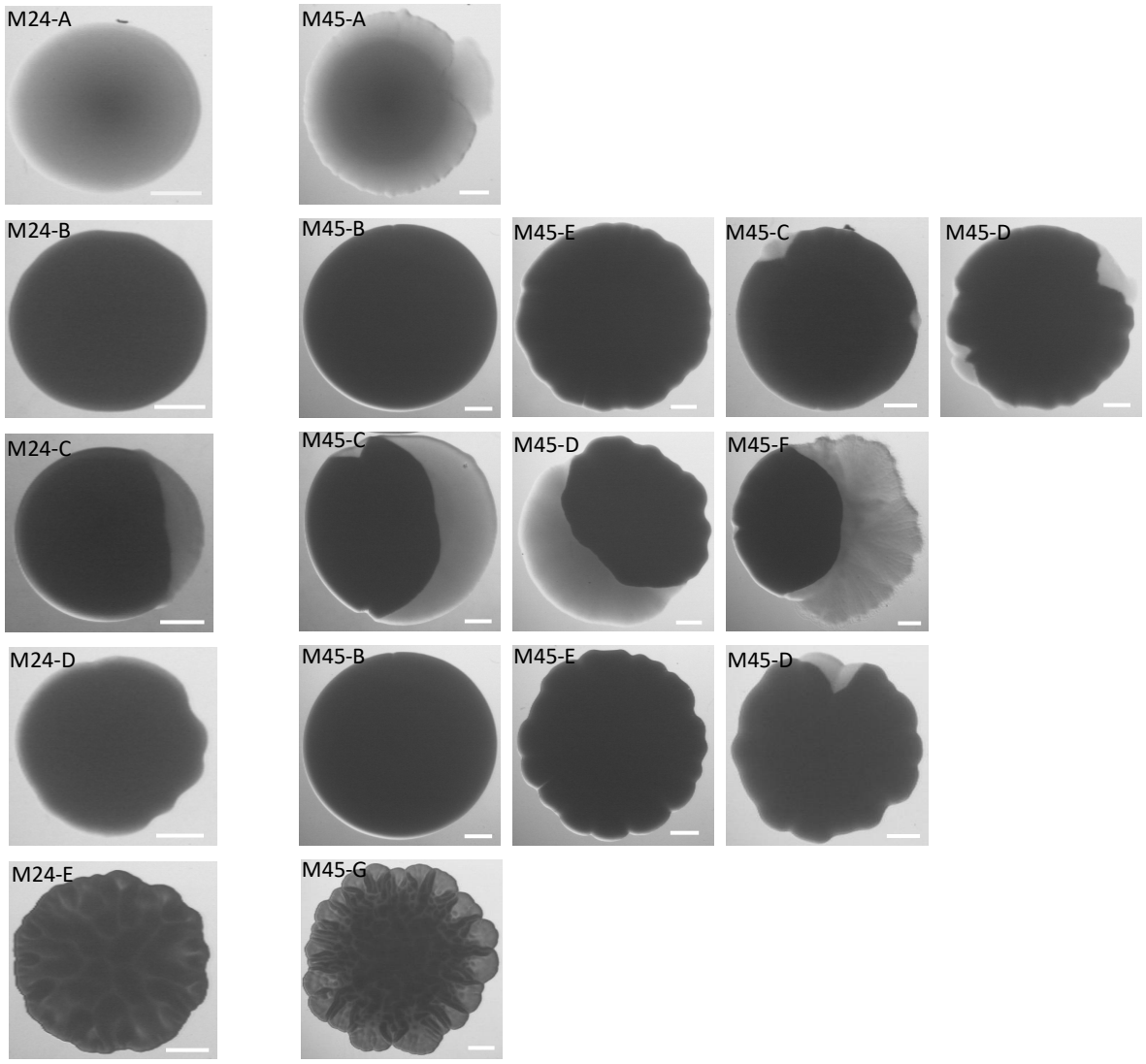


Figure 6- Morphologic features of planktonic *K. pneumoniae* isolates colonies observed on TSA, at 24 hours of incubation and the colony morphology development of the variants identified after 45 hours of growth. All morphotypes were observed at least 2 times of the 3 performed. Each white bar represents 1 mm.

The incubation period was determined according to the literature on phenotypic variability[149], although the data on *K. pneumoniae* were scarce.

According the morphological criteria, 5 morphotypes were identified in a 24 h incubation period, while in a 45 h incubation period 7 morphotypes were identified, as shown in the appendix (Table 4.2). At 24 h of colony growth, 5 different colony morphotypes were observed (Figure 6) exhibiting different colony traits

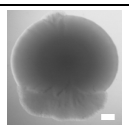
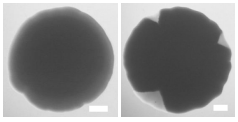
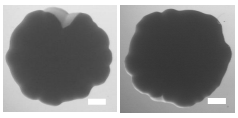
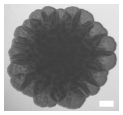
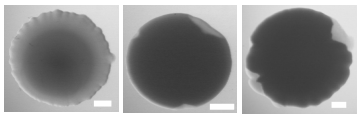
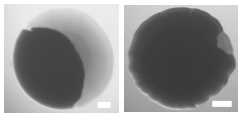
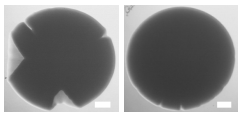
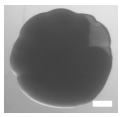
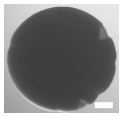
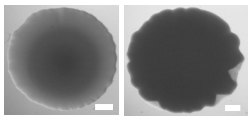
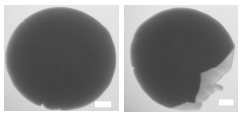
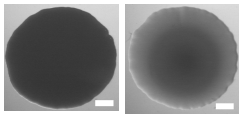
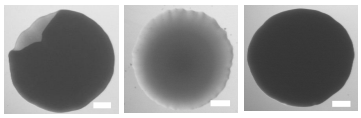
(Table 4.2). However, after a total of 45 h of colony growth, 7 colony morphotypes were detected. This result demonstrated that the same colony morphotype detected at 24 h of colony growth can evolve to different colony morphotype at 45 h. After 45 h of growth the colonies may remain similar or can completely change their appearance becoming a totally different morphotypes. For instance, the colony morphotype named M24-D after a total of 45 h evolve to different 3 morphotypes in a strain-dependent way. Most clinical isolates studied herein of *K. pneumoniae* have more than one different morphotype, except for the ESBL-producing strains 4, 9, 67, 95 and the susceptible strain ATCC 11296.

Taking into account the observed changes and increased morphological diversity from 24 to 45 h of growth, the optimal colony growth time was estipulate as 45 h. At this time, the analysis did not loss important information about clonal diversity that was not observed at 24 h of growth. Therefore, the subsequent analysis was only referred to colonies detected after 45 h of growth.

Population diversity of clinical isolates grown as planktonic culture was determined based on the number of morphotypes identified. Each of the colonies formed by the clinical isolates of *K. pneumoniae* were classified according to the characteristics described in Table 3.1 and the results are in the Table 4.3, of the appendix.

All *K. pneumoniae* strains exhibited colonies with a different number of morphotypes, ranging from 1 to 3 (Figure 7). The susceptible strain ATCC 11296 has only one morphotype, which is unchanged from the morphotype that appeared at 24 h, the same as for the isolate 95. The appearance of the colonies of isolates 9, 66 and 67 also resumed to only one morphotype, however different from what appeared at 24 h, with phenotypic changes occurring over the incubation period. Most of the remaining *K. pneumoniae* isolates had 2 different morphotypes, which did not appear to be influenced by their resistance profile as it was so variable. One can look at isolates 57, 66 and 68, each of which has 3, 1 and 2 different morphotypes, respectively.

The morphotypes identified differ essentially in two parameters, the margin and opacity. Interestingly, the opacity is one of the most relevant factors regarding the characterization of the isolates, as there was a great variety among them, even more the existence of colonies that present a mixed opacity. The less frequent change has to do with the characterization of the form, texture, consistency and elevation.

K. pneumoniae Strain	Colony Morphotype
ATCC 11296	
Isolate 1	
Isolate 4	
Isolate 9	
Isolate 57	
Isolate 63	
Isolate 64	
Isolate 66	
Isolate 67	
Isolate 68	
Isolate 69	
Isolate 70	
Isolate 71	

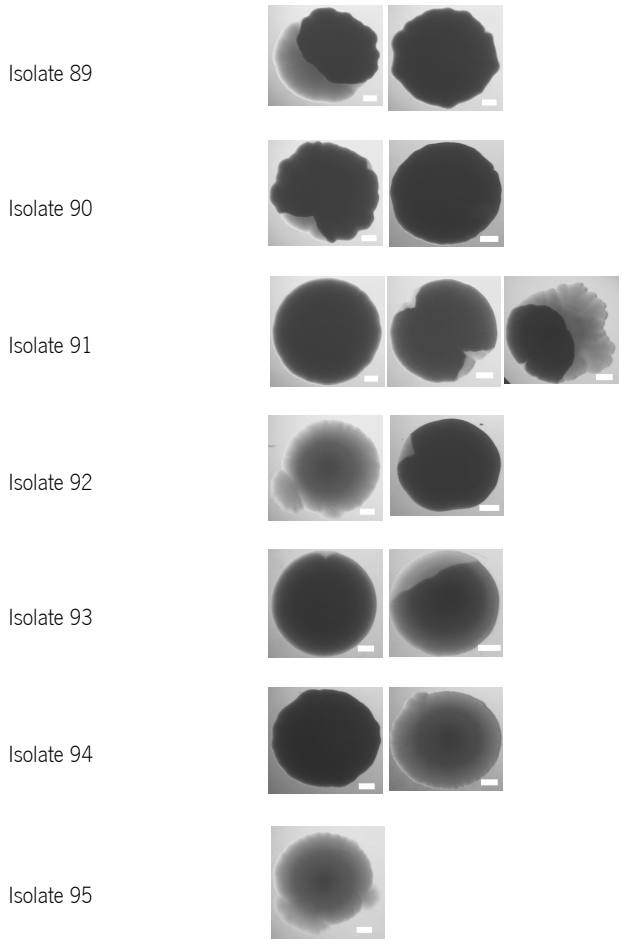


Figure 7- Appearance of colony morphology of *K. pneumoniae* clinical isolates growth in a planktonic culture for 45 hours and plated in TSA. White bars represent 1mm.

Clonal diversity in *K. pneumoniae* has been a poorly explored subject, however investing in the knowledge of this feature is of great importance. Despite the variable number and distinct colony morphologies observed, there did not seem to be a relationship between these factors and the resistance profile of *K. pneumoniae* isolates. The identified morphotypes may be related to different characteristics associated with these strains other than their ability to resist antibiotics and may interfere with their pathogenicity and especially the biofilm formation capacity.

4.2.3. Phenotypic detection of biofilm formation

The capacity of biofilm formation of *K. pneumoniae* is one of the major contributors for its increased pathogenic potential. The most notorious characteristic of biofilms is their resistance against the action of antimicrobial agents due to bacterial growth conditions[130]. Comparing with other species such *P. aeruginosa, S. aureus, S. epidermidis, E. coli*, the understanding about the ecology and physiology of *K. pneumoniae* biofilms is still limited. Therefore, a significant effort was done in this study to provide insights about biofilm physiology.

Because of the high number of *K. pneumoniae* strains used in this study, a qualitative study (Congo red agar method) about the ability of bacteria to form biofilm formation was first performed. The results demonstrated that the majority 55 % (11) of *K. pneumoniae* strains were able to form biofilms. However, this qualitative method exhibited significant limitations.

According the method describe by Nachammai *et al.*[138], colonies should exhibit black or red color meaning that bacteria can form biofilms or not, respectively. During this experiment, grey and pink colonies were observed (Figure 4.1 of the appendix), which is not described by Nachammai *et al.*[138]. Therefore, 40 % (8) of *K. pneumoniae* strains had their ability to form biofilms indeterminate.

Table 3. Results from the Congo red agar method used to determinate the biofilm formation capacity of *K. pneumoniae* clinical isolates and *K. pneumoniae* ATCC 11296.

K. pneumoniae strains	Colony color	Biofilm Production
ATCC 11296	Red	Negative
Isolate 1	Black	Positive
Isolate 4	Black	Positive
Isolate 9	Black	Positive
Isolate 57	Black	Positive
Isolate 63	Black	Positive
Isolate 64	Grey/Pink	Indeterminate
Isolate 66	Grey/Pink	Indeterminate
Isolate 67	Grey/Pink	Indeterminate
Isolate 68	Black	Positive
Isolate 69	Grey/Pink	Indeterminate
Isolate 70	Black	Positive
Isolate 71	Grey/Pink	Indeterminate
Isolate 89	Black	Positive
Isolate 90	Grey/Pink	Indeterminate
Isolate 91	Black	Positive
Isolate 92	Black	Positive
Isolate 93	Black	Positive
Isolate 94	Grey/Pink	Indeterminate
Isolate 95	Grey/Pink	Indeterminate

According to the results, ATCC 11296, the only antibiotic susceptible strain, developed red colonies which led to its classification as a non-biofilm producing strain. Due to the limitations of this methodology

in characterizing such important virulence factor further a qualitative method was applied to infer the biofilm formation of these strains.

4.3. Biofilm Formation

As noted above, biofilm formation capacity is one of the most important virulence properties in bacteria. Due to the difficulty in classifying *K. pneumoniae* strains as biofilm-producers in the previous section, here the biofilm formation capacity was characterized by a quantitative test, the CV method. Twenty-one *K. pneumoniae* clinical isolates were analyzed, including resistant clinical isolates, 16 producers of ESBLs and 3 producers of carbapenemases and 2 susceptible to antimicrobial agents isolates (isolates 2 and 3), and the reference strain ATCC 11296.

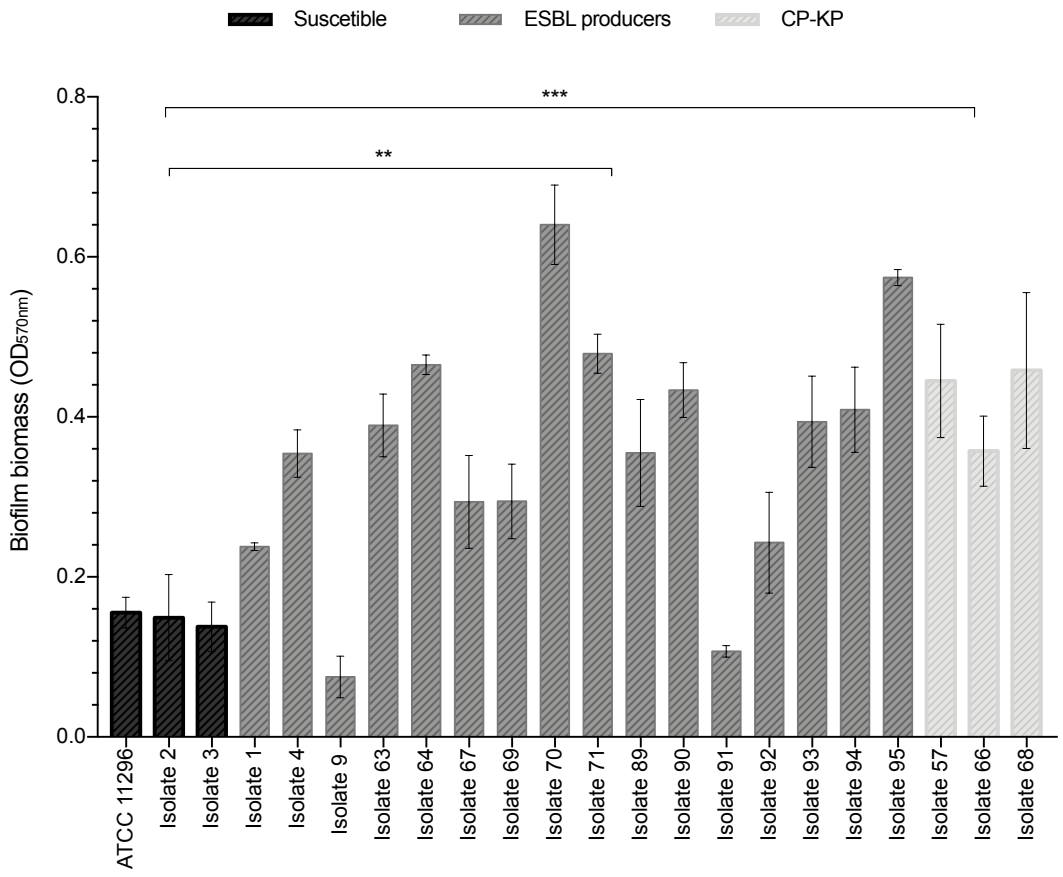


Figure 8- Biofilm formation capacity of *K. pneumoniae* isolates and ATCC 11296. The results were obtained though the CV method and the results are given in optical density of 570 nm. Bars represent the average of biofilm mass from three independent experiments for each isolate tested. Error bars indicate the standard deviation. Data was analyzed by Turkey's multiple comparison test and (*) represent the statistically significant differences between the groups (** $p<0,01$; *** $p<0,001$).

The results demonstrate that *K. pneumoniae* isolates had a diverse ability to form biofilms (Figure 8). In general way, susceptible strains (ATCC, isolate 2 and 3) exhibited reduced ability to form biofilms comparing to the ESBL- and the carbapenemases producing strains. However, there was no rule because also the isolate 9 and 91, both ESBLs producers, exhibited limited ability to form biofilms.

For the remaining isolates, a significant difference in biofilm formation is observed when compared with ATCC 11296 ($p < 0,05$). The biomass variation within the susceptible isolates and ATCC 11296 group is not significant, as is the variation between the carbapenemase producing isolates (CP-KP), however in the group of ESBL-producing strains there is a significant variability in the biofilm production capacity among the isolates.

Regarding the biomass quantification between isolates with different resistance profiles, a statistically significant difference ($p < 0,01$) was observed between the susceptible strains group and the ESBL-producing strains, and this variation increased when susceptible strains were compared to CP-KP ($p < 0,001$). The results allow us to conclude that in fact, there appears to be a relationship between biofilm formation capacity and the resistance profile of clinical isolates of *K. pneumoniae*, and resistant strains tend to form a higher amount of biomass. The results are corroborated by other studies in which there was a direct relationship between biofilm production and resistance to antimicrobial agents[102,150,151]. The explanation for this relationship is discussed by some authors. Vuotto *et al.* argues that under antibiotic pressure, especially at a subinhibitory concentration, bacteria tend to increase their biofilm production capacity[102], probably as a defense mechanism against antimicrobial action. On the other hand, Hennequin *et al.* have proposed that some plasmid-encoded resistance genes lead to increased fimbrial gene expression, and consequently biofilm formation[151].

Since the data of biofilm formation was retrieved from CV staining, it may be suggested that the isolates of *K. pneumoniae* with the highest biofilm formation capacity could be constitute with increased matrix. CV is one of the most popular method of biofilm characterization and it is based on the ability of this dye to stains the whole biofilm biomass, including both live and dead cells as well as the biofilm matrix. Therefore, it is possible to infer about the amount of the exopolysaccharide matrix without removing the biofilm from the microtiter plate[152].

4.3.1. Composition of the extracellular polymeric matrix

The extracellular matrix of biofilms constitutes a hallmark of biofilms and one of the major responsible for antibiotic tolerance. The biofilm matrix provides a physical and chemical barrier protecting bacteria against antibiotics, host immune system molecules and dehydration[153]. The extracellular matrix is

fundamentally constituted by polysaccharides, proteins, nucleic acids and lipids[154]. Therefore, it was considered important to infer the composition of this barrier in order to better understand how antimicrobials are neutralized or eliminated within biofilms.

The presence of DNA in the exopolysaccharide matrix is involved in cell adhesion and dissemination of resistance genes[155]. The content of the matrix in proteins and DNA can have a huge influence on the pathogenic potential of *K. pneumoniae* as well as on the potentiation of its resistance to antibiotics, being this one the alarming reasons for the formation of bacterial biofilms both in hospital equipment and in the environment.

In this experiment the clinical isolates of *K. pneumoniae* 66, 70, 90 and 94 were selected because together they represent all the resistance genes present in the sample of *K. pneumoniae* isolates used in this study. In addition, these isolates exhibited increased biofilm formation capacity. In order to evaluate the composition of the matrix of *K. pneumoniae* biofilms, enzymatic dispersion was performed with two different enzymes, DNase I and Proteinase K. 24-h old biofilms were subject to these two enzymatic treatments, separately, for a period of 4 h and then biomass was quantified by the CV method (Figure 9).

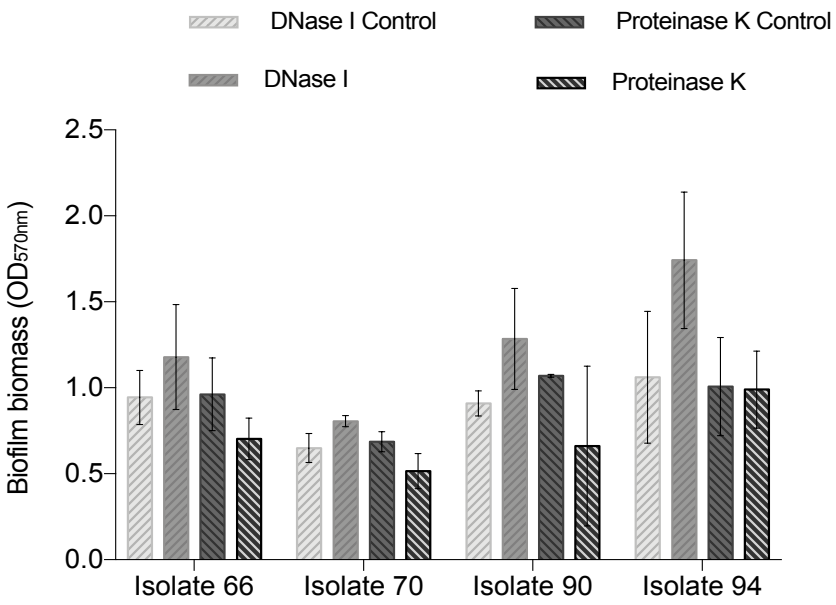


Figure 9- Biofilm biomass before and after enzymatic digestion of *K. pneumoniae* 24h-old biofilms in order to infer part of extracellular polymeric matrix. Biofilms were treated with DNase I and Proteinase K for a 4 hours period. Bars represent the average of biofilm mass from three independent experiments for each isolate tested. Error bars indicate the standard deviation.

The enzymatic digestion by proteinase K provoked a reduction in biofilm biomass in contrast to DNase I (Figure 9). This result demonstrated that DNA was absent or limited in the matrix of *K. pneumoniae* biofilms. Similar results were obtained by Bandeira *et al.,* who verified an ineffective action of DNase I on *K. pneumoniae* biofilms, concluding that the matrix of the analyzed biofilms contained mostly proteins and

exopolysaccharides instead of nucleic acids[139]. However, Singh *et al.*[154] obtained opposite results. In this latter study, the treatment of biofilms formed by clinical isolates of *K. pneumoniae* provoked a biomass decrease of around 28 %, demonstrating that the matrix definitely contained DNA.

The presence of extracellular DNA in the exopolysaccharide matrix is a determining factor in the development of biofilm because it assists cell adhesion to surfaces, it is also responsible for maintaining the structural integrity of this complex[155]. Nevertheless, extracellular DNA plays an extremely important role in the pathogenicity of bacteria. Initially, it provides protection against the host immune system and enhances antimicrobial resistance as it triggers the expression of genes that increase the ability to resist antibiotics[155]. The absence of extracellular DNA found in the results may indicate some fragility of *K. pneumoniae* biofilms. Furthermore, the presence of extracellular DNA in biofilms provides for horizontal gene transfer through transformation, which corroborates the status of biofilms creating the ideal environment for the spread of antibiotic resistance[155]. The matrix of the clinical isolates of *K. pneumoniae* was not rich in extracellular DNA, which may influence the resistance transfer ability of these isolates.

Regarding the data of proteinase K digestion, it seemed that the matrix of *K. pneumoniae* biofilms had a protein content in its constitution. After administration of this protease against biofilms, it was achieved about 25 % of reduction in 3 (isolates 66, 70 and 90) of 4 isolates. A reduction of 1 % was observed in the biofilm biomass of the isolate 94. The efficacy of proteinase K over biofilm matrix was described previously and increased reduction rates ranging from 65 to 69 %[156,157] were reported. The different biofilm mass reduction may due to the different biofilm formation conditions, including biofilm age. In this study, biofilm matrix was treated after 24 h of growth, while the other studies performed the enzymatic degradation over 72-h old biofilms. In this last case, the period of surface adhesion and cell growth is much longer, giving rise to a mature biofilm probably with a three-dimensional structure more complex and increased amount of exopolysaccharide matrix. Consequently, the content of protein could be higher and proteinase K digestion rates higher too. Furthermore, the site of infection from which the clinical isolates provided is a factor that may influence the chemical composition of the biofilm matrix. Different infection sites can exert different environmental pressures that bacteria must adapt to survive. According Singh *et al.*[154], *K. pneumoniae* clinical isolates from blood, pus, stool and urine can exhibit different constituents on the matrix, in particular, protein and nucleic acid. For instance, biofilms formed by clinical isolates from pus, stool and blood had increase content of proteins. Urine isolates were found to have only 6,97 % of protein content. In this study, isolates from blood (isolate 70), sputum (isolate 90) and urine (isolate 66) were those that had more proteins in their matrix. Regarding the study by Singh *et al.*[154], the results coincide with respect to clinical blood isolates, however they are contradictory concerning clinical isolates of urine.

4.3.2. Biofilm formation and antimicrobial resistance

To determine the possible correlation between biofilm formation capacity and resistance of *K. pneumoniae* strains, a statistical analysis was performed using the Kruskal-Wallis test (normality assumption was not accomplished). This test makes it possible to perform an analysis that differentiates biofilm formation capacity between susceptible and resistant strains, and is a test suited to smaller samples. All clinical isolates of *K. pneumoniae* previously classified as resistant, and susceptible strains ATCC, isolates 2 and 3 were used in this section. Due to the reduced sample of susceptible strains, the representativeness of this group is not guaranteed during the analysis (Figure 4.2).

Statistical analysis was performed to correlate biofilm formation with ESBL-producing and non-producing isolates, as well as with the presence of each resistance gene individually (Table 4). The results showed that there was an association between biofilm formation and the production of ESBLs. Surgers *et al.*[158], also demonstrated a relevant association between biofilm formation capacity and ESBL production in clinical isolates of *K. pneumoniae*.

It can be concluded that the presence of certain resistance genes in isolated, double or triple combinations does not influence the biofilm formation capacity, which is intriguing since these are the same ESBL-producing strains used. Indeed, in the analysis of ESBL-producing and non-producing strains, there is a very small sample of non-producing isolates that may not be representative and lead to analysis of these results (Figure 4.2).

Biofilm production provides an important pathogenic advantage, allowing protection against host immune responses and reduced susceptibility to antibiotic therapy. There are a few evidences that this virulence factor can be related to antibiotic resistance in *K. pneumoniae*. A study performed in 2014 analyzed in urine samples from catheterized patients revealed that 80 % of the biofilm-producing strains exhibited a multi-resistant phenotype. Moreover, positive biofilm isolates showed 93,3 %, 83,3 %, 73,3 % and 80 % resistance to nalidixic acid, ampicillin, cefotaxime and co-trimoxazole, respectively, compared to the 70 %, 60 %, 35 % and 60 % resistance shown by biofilm non-producers for the respective antibiotics[102].

Table 4. Differences between ESBL-producing and non-producing strains of *K. pneumoniae* in the capacity of biofilm formation, and the differences between the strains containing different resistance genes, according the Kruskal-Wallis test.

	ESBLs Production		SHV		TEM		CTX		KPC	
	KW H	*p*-value	KW H	*p*-value	KW H	*p*-value	KW H	*p*-value	KW H	*p*-value
Biofilm Formation	4,634	0,031*	1,800	0,235	0,000	1,000	1,152	0,315	0,397	0,591

*significant at level 0,05

The antibiotic resistant *K. pneumoniae* clinical isolates used in this study have not only one resistance gene, but double and triple combinations of genes from different resistance families which makes them MDR. Thus, in addition to the analysis carried out where only the presence or absence of one gene is evaluated individually, it was decided to verify the existence of the same correlation with the biofilm formation but with the conjugations of different genes found in the clinical isolates of *K. pneumoniae*, double and triple (Tables 5 and 6, respectively). The respective boxplots are shown in Figures 4.4 and 4.5 in the appendix.

Table 5. Differences in biofilm formation between the isolates with double combinations of resistance genes, according Kruskal-Wallis test.

	SHV CTX		SHV TEM		KPC SHV		KPC TEM	
	KW H	*p*-value	KW H	*p*-value	KW H	*p*-value	KW H	*p*-value
Biofilm Formation	1,152	0,315	0,037	0,888	1,089	0,368	0,089	0,824

Table 6. Differences in biofilm formation between the isolates with triple combinations of resistance genes, according Kruskal-Wallis test.

	SHV TEM CTX		KPC SHV TEM	
	KW H	*p*-value	KW H	*p*-value
Biofilm Formation	0,083	0,815	0,667	0,588

Regarding the relationship between biofilm formation and the presence of different resistance genes, Ramos-Vivas *et al.*[159] are in agreement with the results of the analysis, since they did not verify any relation between these two factors. On the other hand, Traore *et al.* [127], demonstrated that CTX-M-15-producing *K. pneumoniae*, in the presence of cefotaxime, enhanced the biofilm formation.

These few examples described here highlight the importance to deeply study the interplay between antibiotic resistance and virulence. The understanding about this relationship will significantly impact on the current antimicrobial stewardship and also on microbial diagnosis.

4.3.3. Biofilm formation and clonal diversity

In this section we intended to infer whether or not clonal diversity in the planktonic state may interfere with the biofilm formation capacity of clinical isolates of *K. pneumoniae,* in order to analyze the differences in biofilm formation capacity between strains of 1, 2 or 3 associated morphotypes was used the test of statistical analysis Kruskal-Wallis (Table 7). From the results obtained, it is verified that the biofilm formation capacity was not correlated with the number of morphotypes that each strain presented in planktonic state, observing a great dispersion of the values (Figure 4.6). However, it is important to note that data presented some dispersion and the representativeness of each of the groups may not have been

guaranteed due to the small sample size. Therefore, more experiments should be performed using more *K. pneumoniae* isolates to increase the sample size and consolidate the results obtained.

Table 7. Differences between the number of morphotypes and the ability to form biofilm of *K. pneumoniae* strains, according to Kruskal-Wallis test.

	Diversity	
	KW H	*p*-value
Biofilm Formation	1,396	0,519

Once the relationship between biofilm formation capacity and the diversity of the population analyzed was not verified, it was decided to analyze the existence of a relationship between biofilm formation potential and the characteristics individually attributed to each morphotype presented in Table 4.3. The morphological characteristics of the colonies that dictate the differences between the morphotypes were margin and opacity, so these were the characteristics used for the analysis of association. The other traits had no variability, or the variability was not sufficient to run a statistical test. To determine the role of *K. pneumoniae* strains morphology in biofilm formation, differences in biofilm formation between strains with different margins and different opacities (Table 8) were analyzed according to the Kruskal-Wallis test.

Table 8. Differences in biofilm formation capacity between the *K. pneumoniae* strains with different colony characteristics, using Kruskal-Wallis test.

	Margin						Opacity					
	Entire		Lobate		Undulated		Opaque		Translucent		Opaque and Translucent	
	KW H	*p*-value	KW H	*p*-value	KW H	*p*-value	KW H	*p*-value	KW H	*p*-value	KW H	*p*-value
Biofilm Formation	0,353	0,588	0,280	0,631	0,244	0,656	0,006	0,970	3,720	0,057	0,093	0,800

From the statistical results obtained, it was revealed that there were no differences in biofilm formation capacity related to the different margins or opacity of the colonies (Figure 4.7 and Figure 4.8).

Colony diversity may be a phenotypic indicator that predicts the characteristics of a particular strain and may function as an indicator of the behavior of bacteria in a variety of situations, particularly in terms of expression of their virulent potential[106]. In this study no relationship was detected between the morphotype diversity of *K. pneumoniae* isolates and biofilm formation, as well as between individual colony characteristics related to margin and opacity of biofilm and biofilm formation. In fact, the clonal diversity of *K. pneumoniae* is slight studied, making it difficult to understand its importance in the virulent potential of resistant *K. pneumoniae* strains.

4.4. Dissemination of antibiotic resistance in polymicrobial communities

Biofilms provides an excellent environment for their resident members to interchange information, in particular antibiotic resistance information, contributing to the uncontrolled emergence and spread of antibiotic resistant strains worldwide. This mechanism has particular importance cornering the spread of ESBL and carbapenemases producing strains non-*K. pneumoniae*. The transfer of these plasmids encoding ESBLs and carbapenemases between bacteria shows their ability to conjugate and demonstrates their driving role in antibiotic resistance, which leads to the development of MDR or XDR bacteria[2].

The aim of this experiment was to investigate the dissemination of resistance in dual-species biofilm formed by *K. pneumoniae* and *P. aeruginosa* (PAO1). *K. pneumoniae* and *P. aeruginosa* are two biofilm-forming organisms that can coexist very well during infections. They are often found during infections in the urinary and respiratory tract and also burn wounds and associated with foreign bodies[130].

The work consisted in the formation of mixed biofilms and subsequent determination of the number of viable cells, quantification of mixed biofilm mass, evaluated by the CV method, and dissemination of resistance to *P. aeruginosa*. In this study, 3 clinical isolates of *K. pneumoniae* (Isolate 66, 70 and 94) were used due to their characteristics, firstly they are strains with good biofilm formation capacity and, secondly, due the fact that this isolates represent all the genetic families present in the total sample of *K. pneumoniae* isolates.

4.4.1. Dissemination of resistance to *P. aeruginosa* in 24-h old mixed biofilms

Before the evaluation of the dissemination of resistance from *K. pneumoniae* to *P. aeruginosa* a brief characterization of the mixed biofilms was made to investigate the kind of interspecies interaction established between these two species. Competitive interactions between bacteria could not favor crosstalk between species and dissemination of resistance could be limited or even inhibited. Based on the results (Figure 10), *K. pneumoniae* to *P. aeruginosa* seemed to well co-existed in a 24-h old biofilms in similar proportions of viable cells (Figure 10B).

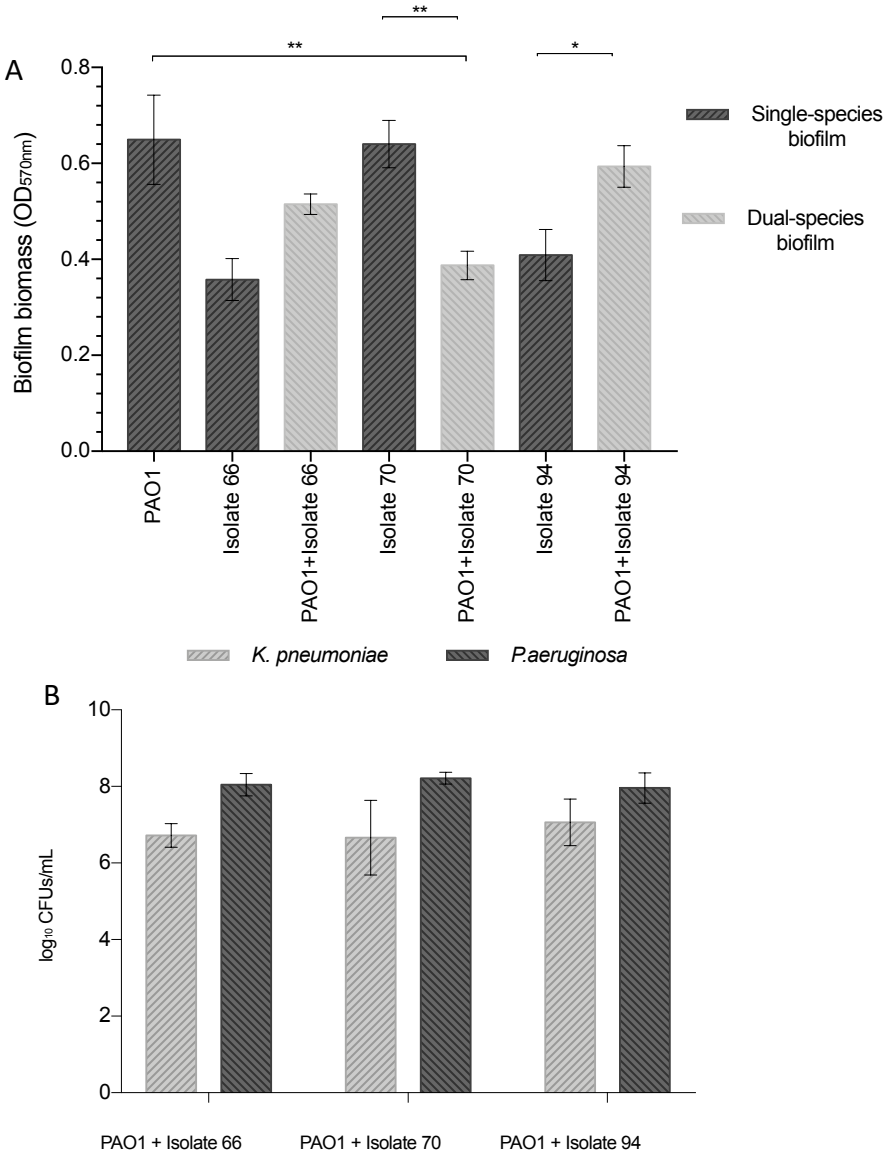


Figure 10- A) Graphical representation of the biofilm formation capacity of PAO1, *K. pneumoniae* isolates, *K. pneumoniae* isolates in consortia with PAO1, for 24 hours. The results were obtained through the CV method and the results are given in optical density of 570 nm. Bars represent the average of biofilm mass from three independent experiments for each isolate tested. Error bars indicate the standard deviation. Data was analyzed by Tukey's multiple comparisons test and (*) represent the statistically significant differences between the groups (* p<0,05; ** p<0,01). B) Counting of the number of cultivable cells from the mixed biofilms of *K. pneumoniae* strains and PAO1, in $\log_{10}$ CFUs/mL. Bars represent the average of three independent experiments for each mixed biofilm tested. Error bars indicate the standard deviation.

From the graph (Figure 10. A) it can be concluded that PAO1 is the most biofilm-capable strain. When compared to single-species biofilm production of *K. pneumoniae* isolates a statistically significant difference with isolates 66 and 94 is remarkable ($p < 0,01$), whereas for isolate 70 there are no significant differences in biomass production. By comparing the biomass formation of single isolates with mixed biofilms, it was possible to verify a tendency for increased biofilm formation in the case of isolate 66 not significant ($p > 0,05$) but with some significance in isolate 94 ($p < 0,05$). Contrarily, isolate 70 showed a decrease in biomass formation capacity when in consortium with *P. aeruginosa* strain ($p < 0,01$). Analyzing PAO1 biofilm formation and comparing with mixed biofilms, only statistically significant differences are seen in relation to

the consortium with isolate 70 ($p < 0,01$). Regarding the number of viable cells, it was possible to affirm that the two species coexist together very well, since a very close cellular concentration was observed after biofilm formation. These results seem to indicate that in fact, there are no inhibitions on growth by any of the species.

Verifying the co-existence of both bacterial species in mixed biofilms, dissemination of resistance of *K. pneumoniae* to PAO1 was investigated. Cells from the mixed biofilms were collected and plated in LB medium and LB medium with 4 μg/mL of ceftazidime (CAZ) (Figure 11). Since the clinical isolates of *K. pneumoniae* used are resistant to this antibiotic, it would be expected their growth in this medium. On the other hand, PAO1 is susceptible to this antibiotic belonging to the class of cephalosporins, and for this reason, its growth was not presumed. Detection of *P. aeruginosa* in this medium might indicate its acquisition of resistance due to contact during the growth on biofilm with *K. pneumoniae*, also becoming a CAZ resistant strain.

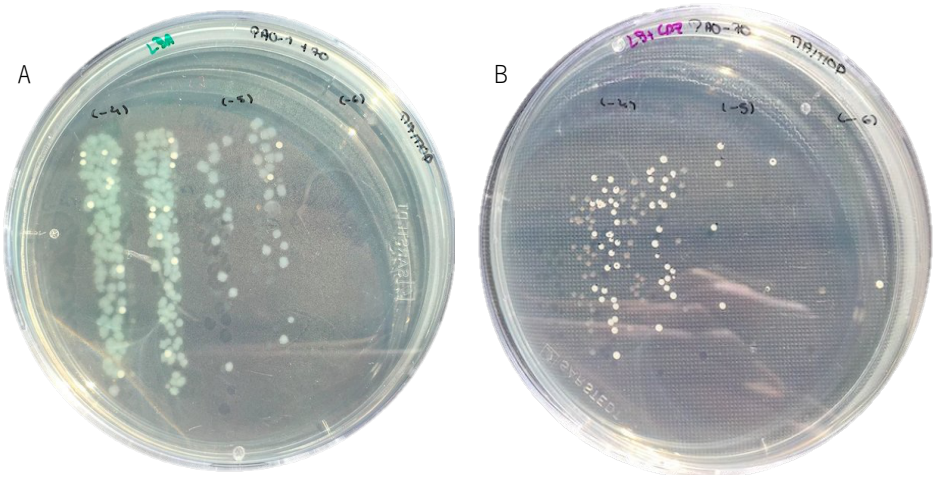


Figure 11- Dissemination of resistance from *K. pneumoniae* (Isolate 70) to PAO1. A) Cells from mixed biofilm plated in LB medium. In the image it is possible to observe the growth of both species. B) mixed biofilm cells plated in LB medium supplemented with 4 μg / mL CAZ. In this case it is possible to observe the growth of *K. pneumoniae* and the absence of PAO1.

Figure 11 illustrates the growth of cells harvested from the mixed biofilm of *K. pneumoniae* isolate 70 with PAO1, however it is representative of the results obtained in the remaining analyzed biofilms. The absence of *P. aeruginosa* growth in LB agar medium with CAZ pointed to the non-dissemination of resistance to CAZ from *K. pneumoniae* to *P. aeruginosa*. Indeed, the number of cultivable cells determined seemed to indicate the *P. aeruginosa* eradication after the application of 4 μg/mL of CAZ (Figure 12).

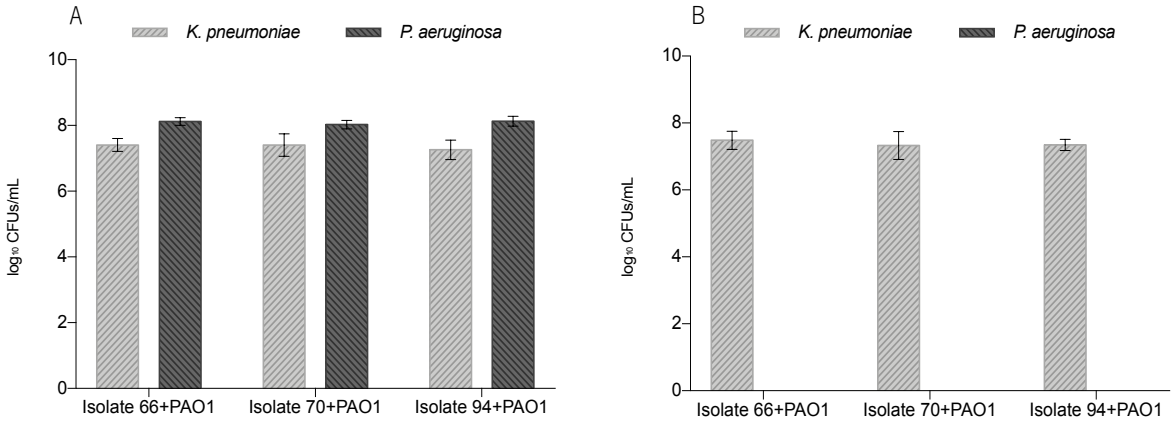


Figure 12- Number of cultivable dual-species biofilm-cells of *K. pneumoniae* strains and PAO1, in log_{10}CFUs/mL. A) Cells from mixed biofilm that were plated in LB medium. B) Cells from mixed biofilm that were plated in LB medium supplemented with 4 μg/mL CAZ. Bars represent the average of three independent experiments for each mixed biofilm tested. Error bars indicate the standard deviation.

In medium supplemented with antibiotic (Figure 12.B), the results previously obtained by plating the cells were confirmed. *K. pneumoniae* strains maintained their growth and high cell concentrations as they are resistant to CAZ and the presence of the antibiotic did not impair their growth. On the other hand, there was an absence of *P. aeruginosa*. The results point to the non-detection of *P. aeruginosa* resistant to CAZ.

4.4.2. Dissemination of resistance to *P. aeruginosa* in 72-h old mixed biofilms

Once the spread of CAZ resistance between the two bacterial species was not detected in a 24-h old biofilm, it was hypothesized whether it would occur in a more mature biofilm. Thus, the resistance dissemination assays were performed using a 72-h old mixed biofilm. As previous, cells harvested from mixed biofilms were plated in LB medium and LB supplemented with 4 μg / mL CAZ. The results are shown in Figure 13, illustrating the results obtained for the mixed biofilm composed of isolate 94, however is representative of the results obtained with the remaining *K. pneumoniae* isolates. In the medium supplemented with antibiotic, *P. aeruginosa* growth was not detected, which seems to indicate that there was no resistance spread among the species.

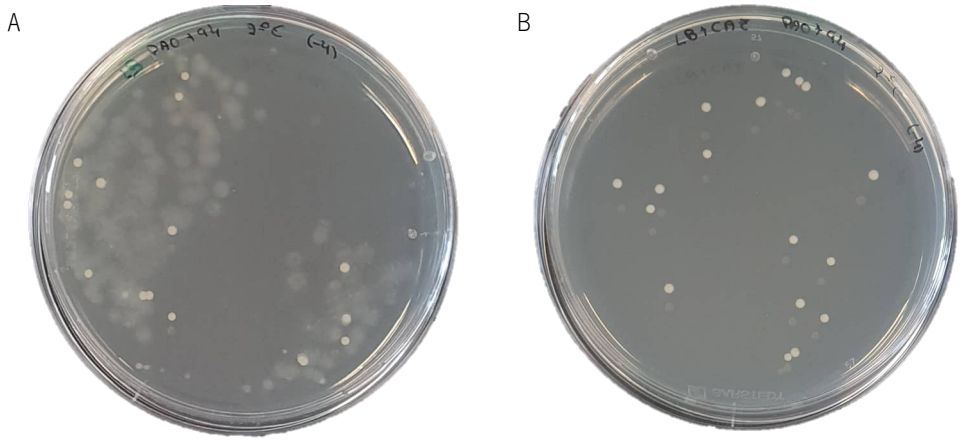


Figure 13- Dissemination of resistance from *K. pneumoniae* (Isolate 94) to PAO1. A) Cells from mixed biofilm plated in LB medium. In the image it is possible to observe the growth of both species. B) mixed biofilm cells plated in LB medium supplemented with 4 μg / mL CAZ. In this case it is possible to observe the growth of *K. pneumoniae* and the absence of PAO1.

Similarly, 72h-old mixed biofilms were subject to a 4 μg / mL of CAZ during 24h. Ending the antibiotic treatment, the number of viable cells of each species was determined.

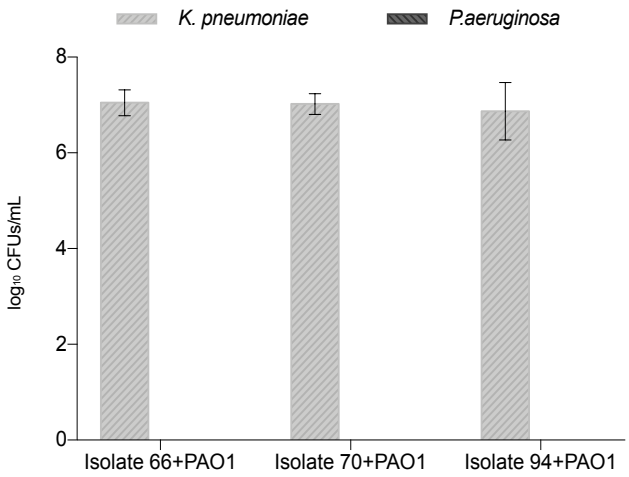


Figure 14- Number of cultivable dual-species biofilm-cells of *K. pneumoniae* strains and PAO1, in $\log_{10}$ CFUs/mL. Cells from mixed biofilm that were plated in LB medium supplemented with 4 μg/mL CAZ. Bars represent the average of three independent experiments for each mixed biofilm tested. Error bars indicate the standard deviation.

Regarding the number of viable cells (Figure 14), the results were similar to those obtained previously in the study of the dissemination of resistance in biofilms of 24 h. Even in a mature biofilm, it seemed that *K. pneumoniae* resistance to CAZ did not disseminate to *P. aeruginosa* because no viable cells were detected on solid media supplemented with antibiotic (Figure 14).

In polymicrobial consortia, the different species that constitute them may be organized in diverse ways: mono-species microcolonies, co-aggregation and arrangement in layers[130]. In mono-species colonies each species forms individual structures, which implies the absence of commensal inactions. In co-aggregation

microorganisms are also dispersed throughout the biofilm, so different species can interact with each other. In layering, species can interact with each other and act synergistically, or compete with each other[160]. Dual-species biofilms of *K. pneumonia* and *P. aeruginosa* have a layered distribution pattern. Due to its high colonization capacity, *P. aeruginosa* forms the base structure while *K. pneumoniae* forms a tower structure at the top[161]. According to Elias *et al.*[160], the spatial distribution of species in mixed biofilms can greatly alter gene expression during biofilm formation and consequently the interactions that occur between species, namely the interactions that promote the transmission of resistance by horizontal gene transfer. In the present study, it was not verified the dissemination of resistance to the CAZ of *K. pneumoniae* for *P. aeruginosa*, which may be related to the disposition of the species in the biofilm, this is another factor that adds complexity to the possible interactions within the community.

Indeed, the extracellular DNA of the exopolysaccharide matrix of biofilms plays an influential role in the spread of resistance between species, essentially in the bacterial transformation process[155]. As analysed in previous sections, the *K. pneumoniae* biofilms presented low content of extracellular DNA in the matrix, which reinforce the lack of resistance dissemination to *P. aeruginosa*.

4.4.3. Mixed biofilm resistance against ceftazidime

The spread of antimicrobial resistance to *P. aeruginosa* was not detected, and thus it was hypothesized whether *K. pneumoniae* can provide protection to *P. aeruginosa* against antibiotics when they live within biofilms. Resistance acquisition is not only about the exchange of genetic material, but also about the ability of the population to communicate and cooperate with each other so that they can survive to the exposure to antimicrobial agents. Therefore, 24h-old mixed biofilms were subjected to CAZ for 24 h. The *K. pneumoniae* and *P. aeruginosa* strains used were the same of the previous experiments. The mechanism of action of CAZ is the interference of the peptidoglycan system and its use is usually associated with bacteria of the Enterobacteriaceae family, however, this antibiotic has a functional group that guarantees it a greater activity on *P. aeruginosa*, which gives it an advantage over other cephalosporins[162]. In this section, as in the previous one, clinical isolates of *K. pneumoniae* 66, 70 and 94 and PAO1 were used.

First, it was determined the concentration of antibiotic capable of reducing or eradicating PAO1 single biofilms, so that we know about the extent of its action on this strain. For that, 24-h old PAO1 biofilms were treated with 2 mg/L and 8 mg/L CAZ for 24 h and at the end, viable cells were determined, and biofilm biomass was measured by the CV method (Figure 15).

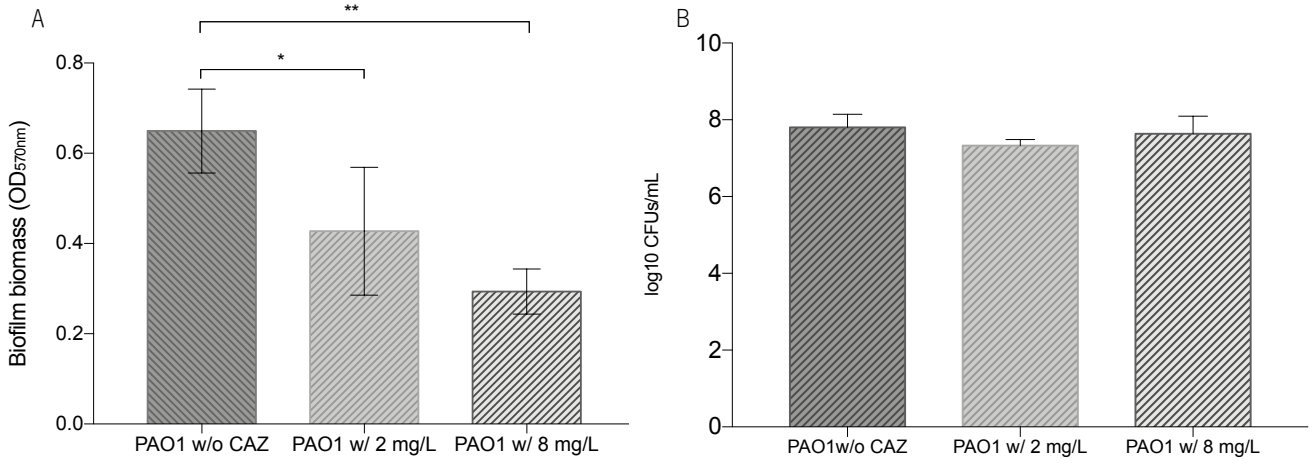


Figure 15- A) Biofilm formation capacity of PAO1 without any treatment and treated with 2 mg / L and with 8 mg / L of CAZ for 24 hours. The results were obtained through the CV method and the results are given in optical density of 570 nm. Bars represent the average of three independent experiments. Error bars indicate the standard deviation. Data was analyzed by Dunnett's multiple comparisons test and (*) represent the statistically significant differences between the groups (* $p < 0,05$; ** $p < 0,01$). B) Cells from PAO1 biofilms without any treatment, PAO1 treated with 2 mg / L of CAZ and PAO1 treated with 8 mg / L of CAZ for 24 hours. Bars represent the average of three independent experiments for each mixed biofilm tested. Error bars indicate the standard deviation.

The use of a 2 mg/L of CAZ in the treatment of PAO1 biofilms provoked a significant reduction in the biomass quantification ($p < 0,05$), being this reduction increased, approximately 50 %, when a treatment of CAZ of 8 mg/L was applied ($p < 0,01$). Interestingly, the number of viable cells of PAO1 biofilms did not seem affected by the antibiotic treatments. The results indicated that CAZ treatments mainly affected the biofilm matrix than provoked cell killing.

Based on the results, 8 mg/L CAZ was the treatment chosen to subject the mixed *K. pneumoniae* and PAO1 biofilms, since it would allow a better evaluation of the protective capacity of *K. pneumoniae*. After the CAZ treatment applied to the 24-h old mixed biofilms, biomass and viable cells were determined.

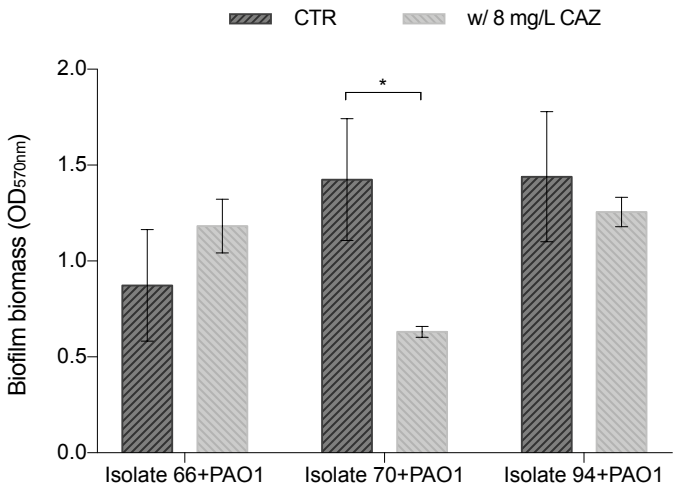


Figure 16- Biofilm biomass of 24-h old mixed biofilms of *K. pneumoniae* and *P. aeruginosa*, with and without CAZ treatment for 24 h. The results were obtained through the CV method and the results are given in optical density of 570 nm. Bars represent the average of three independent experiments for each mixed biofilm tested. Error bars indicate the standard deviation. Data was analyzed by Sidak's multiple comparisons test and (*) represent the statistically significant differences between the groups (* $p < 0,05$).

The results of biofilm biomass quantification revealed that only the mixed biofilm composed by *K. pneumoniae* isolate 70 with PAO1 suffered a significant reduction of biomass ($p < 0,05$) (Figure 16). However, this reduction was minor comparing to that suffered by the single-species biofilms of PAO1 (Figure 15.A). The other two mixed biofilms seemed to be unaffected by the CAZ treatment.

The biofilm constituted by the clinical isolate of *K. pneumoniae* 66, is the only one that presented an increase in biomass formed as if the action of the antibiotic had no effect on the biofilm structure.

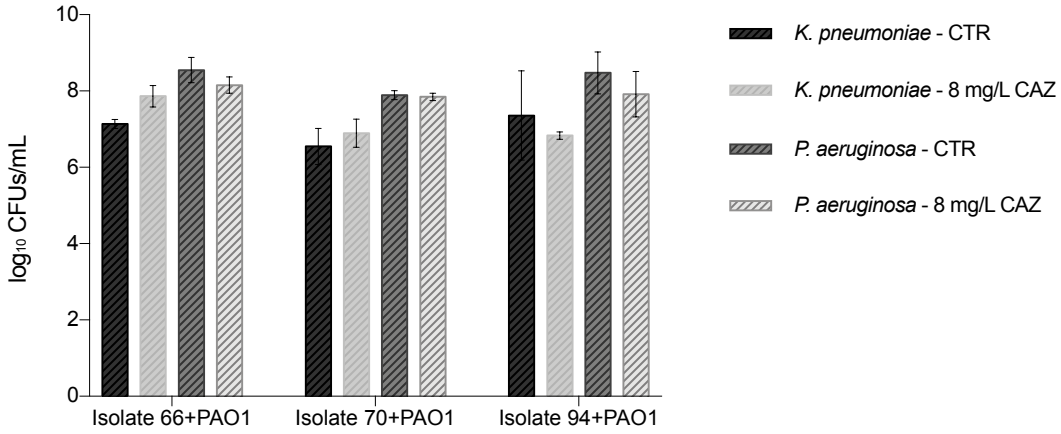


Figure 17- Number of cultivable dual-species biofilm-cells of *K. pneumoniae* strains and PAO1, in log_{10}CFUs/mL. Bars represent the average of three independent experiments for each mixed biofilm tested. Error bars indicate the standard deviation.

In terms of number of viable cells, it seemed that CAZ treatment did not affect both bacterial species because no differences were detected between biofilm treated and no treated with CAZ (Figure 17). These results reinforced that CAZ had action against the biofilm matrix than killing cells.

Taking together the results, *K. pneumoniae* seemed somehow confer protection to *P. aeruginosa* against antibiotic action, but this protection appears to be strain-dependent, as one of the ESBL-producing strains protects PAO1 and one does not. In order to be able to conclude on the relationship between the susceptibility profile of clinical isolates and protection against CAZ, the use of a sample would be necessary.

The development of mixed biofilms in infections is one of the most worrying issues due to the synergistic interactions that may exist between the coexisting species, especially in the presence of multi-resistant strains. Resistance acquisition is not only about the exchange of genetic material, but also about the ability of the population to communicate and cooperate with each other so that they can survive exposure to antimicrobial agents. The resistance offered by the biofilm lifestyle may be related to factors such as exopolysaccharide matrix secretion that compromises antibiotic penetration and neutralizes antimicrobial agents through metal chelation[160,163]. The results seem to be in agreement with the literature, since the counting of viable cells allowed to determine cellular concentrations very close to the respective controls, indicating that the action of the used antibiotic mainly affected the biofilm matrix.

Increased resistance to antibacterial agents may be attributed to the composition of the exopolysaccharide matrix, which may vary depending on the formation of a single or mixed biofilm[160]. The literature describes *P. aeruginosa* PAO1 as a strain capable of altering the composition of its exopolysaccharide matrix during the biofilm formation process and may express antibacterial proteins that assist in the destruction of antibiotics. However, the role of exopolysaccharide matrix components is not as elucidating in the case of mixed biofilms.

5. Conclusions

Throughout this study different properties of the isolates of *K. pneumoniae* were evaluated and, therefore, it was possible to reach several conclusions.

Antibiotic susceptibility tests were initially performed to determine antibiotic resistant *K. pneumoniae* strains, including ESBL-producing isolates. The totality of clinical isolates of *K. pneumoniae* producing ESBLs and carbapenemases was then used to study several characteristics that allowed to understand more about the protective capacity of biofilms.

The morphology of planktonic culture-derived colonies of clinical isolates of *K. pneumoniae* was analyzed in this work. The results allowed to observe an increase of clonal diversity of isolates between 24 and 45 hours of growth, however this factor was not dependent on the resistance profile of the clinical isolates of *K. pneumoniae* analyzed.

Subsequently, the biofilm formation was quantified due to the enormous importance of this factor in the dissemination of resistance between species and increased permanence of bacterial infections. It was possible to conclude that in fact the biofilm formation capacity is increased in the ESBL-producing strains, as previously mentioned in the literature. Conversely, there was no relationship between biofilm formation and colony characteristics of the analyzed strains.

In this work the composition of exopolysaccharide matrix from biofilms of the clinical isolates was also inferred by enzymatic treatment with DNase I and proteinase K, and in the isolates analyzed there was a greater reduction of biofilm formation during treatment with proteinase K, which in leads to the conclusion that the exopolysaccharide matrix of the isolates is mainly composed with a significant amount of proteins. The protein composition of the biofilm matrix is one of the major factors influencing the protection of bacteria involved in a biofilm, as it increases its resistance to penetration by antimicrobial molecules.

The permanence of *K. pneumoniae* in mixed infections and the spread of resistance to other species led to the study of the resistance to CAZ resistance to *P. aeruginosa* in mixed 24 and 72-hour biofilms, however there was no transfer of resistance. In part, due to the biofilm maturation time, because a longer incubation period might be necessary and due to the matrix composition determined above, which was shown to be poor in extracellular DNA, this may have made it difficult to dissolve resistance between species.

Since there was no dissemination of resistance from *K. pneumoniae* to *P. aeruginosa,* it was decided to study whether *K. pneumoniae* in turn provided protection against *P. aeruginosa* antibacterial action. The results showed that *K. pneumoniae* seemed to confer protection to *P. aeruginosa* against ceftazidime, independently of the antimicrobial susceptibility profile of *K. pneumoniae*, but strain-dependent. The cell viability of both species was not compromised but there was a decrease in the formation of mixed biofilms,

which indicates that the matrix of *K. pneumoniae* biofilm seemed to be the responsible for dual-species persistence.

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Appendix

Table 3.1. Morphological features used to characterize *K. pneumoniae* colonies.

Class	Sub-Class
Colony Form	Circular Filamentous Spindle Rhizoid Elliptical Irregular
Colony Margin	Entire Undulate Erase Lobate Curried Filiform
Colony Texture	Smooth Rough Wrinkled
Colony Color	Beige White Brown Yellow Orange Pink Blue Green Red
Colony Consistency	Mucoid Viscous Brittle Dry Moist
Colony Size [a]	Small Large
Type of Surface	Homogenous surface Heterogenous surface
Elevation	Flat Crateriform Raised Umbonate Convex Pulvinate
Colony Opacity	Opaque Iridescent Translucent Transparent

[a] < 3 mm small; > 3 mm large

Table 4.2. Description of morphological characteristics observed at 24 and 45 hours of incubation. * colonies were considered large if presented diameter above 3 mm[149].

		Form	Margin	Texture	Type of Surface	Consistency	Elevation	Opacity	Sheath	Color	Size*
24 hours	Morphotype A	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large
	Morphotype B	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
	Morphotype C	Circular	Undulate	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
	Morphotype D	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
	Morphotype E	Circular	Undulate	Wrinkled	Homogeneous	Dry	Flat	Opaque	No sheath	Beige	Large
45 hours	Morphotype A	Circular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large
	Morphotype B	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
	Morphotype C	Circular	Undulate	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
	Morphotype D	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
	Morphotype E	Circular	Undulate	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
	Morphotype F	Irregular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
	Morphotype G	Circular	Lobate and Undulate	Wrinkled	Homogeneous	Dry	Flat	Opaque	No sheath	Beige	Large

Table 4.3. Detailed morphological description of the colonies identified in the clinical isolates of *K. pneumoniae*. * colonies were considered large if presented diameter above 3 mm[149].

	Form	Margin	Texture	Type of Surface	Consistency	Elevation	Opacity	Sheath	Color	Size
ATCC 11296										
Morphotype I	Circular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large
Isolate 1										
Morphotype I	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Morphotype II	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 4										
Morphotype I	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Morphotype II	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Isolate 9										
Morphotype I	Circular	Lobate/Undulate	Wrinkled	Homogeneous	Dry	Flat	Opaque	No sheath	Beige	Large
Isolate 57										
Morphotype I	Circular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large
Morphotype II	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Morphotype III	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 63										
Morphotype I	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Morphotype II	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 64										
Morphotype I	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Morphotype II	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 66										
Morphotype I	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 67										
Morphotype I	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 68										
Morphotype I	Circular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large
Morphotype II	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 69										
Morphotype I	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Morphotype II	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 70										
Morphotype I	Circular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large
Morphotype II	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large

Isolate 71										
Morphotype I	Circular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large
Morphotype II	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Morphotype III	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 89										
Morphotype I	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Morphotype II	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Isolate 90										
Morphotype I	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Morphotype II	Circular	Undulated	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 91										
Morphotype I	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Morphotype II	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Morphotype III	Irregular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 92										
Morphotype I	Circular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large
Morphotype II	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 93										
Morphotype I	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Morphotype II	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Translucent/Opaque	No sheath	Beige	Large
Isolate 94										
Morphotype I	Circular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large
Morphotype II	Circular	Entire	Smooth	Homogeneous	Moist	Raised	Opaque	No sheath	Beige	Large
Isolate 95										
Morphotype I	Circular	Lobate	Smooth	Homogeneous	Moist	Raised	Translucent	No sheath	Beige	Large

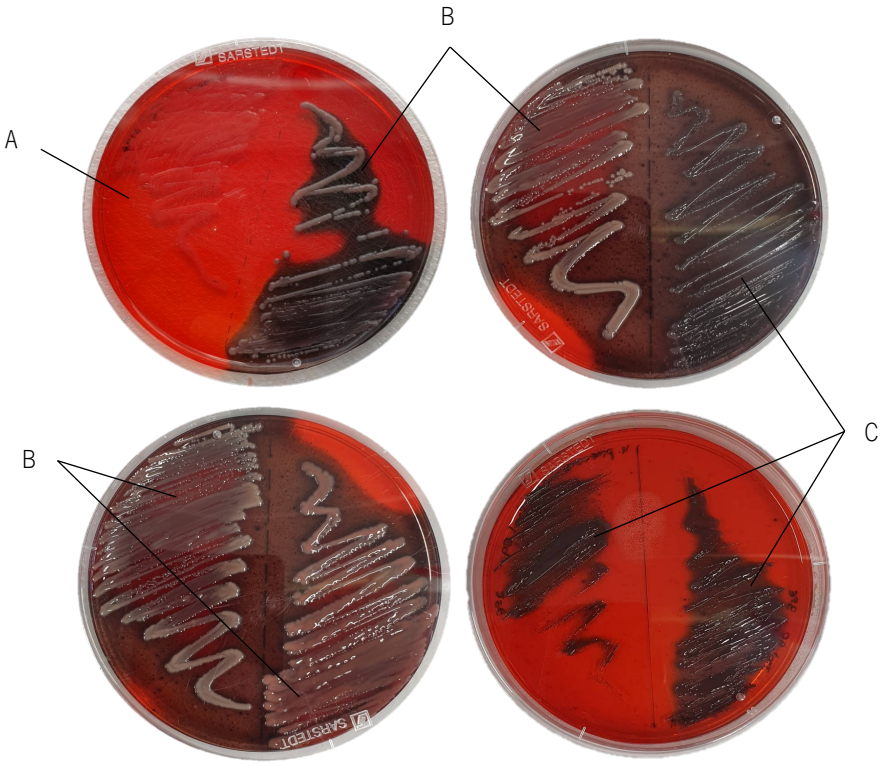

Figure 4.1- Results from the Congo red agar method used to determinate the biofilm formation capacity of *K. pneumoniae* clinical isolates and *K. pneumoniae* ATCC 11296. A: Red colonies indicating non-biofilm producer strains; B: Grey / pink colonies indicating indeterminate strains for biofilm production; C: Black colonies indicating biofilm producer strains.

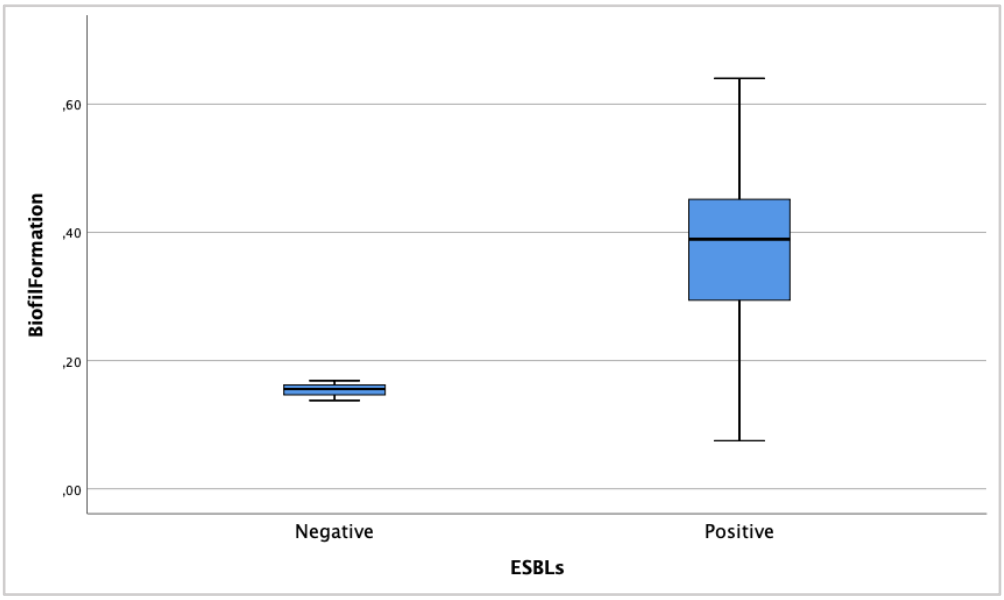

Figure 4.2- Boxplots that display biofilm formation data for ESBLs production. The black bars in the boxes represent the median values.

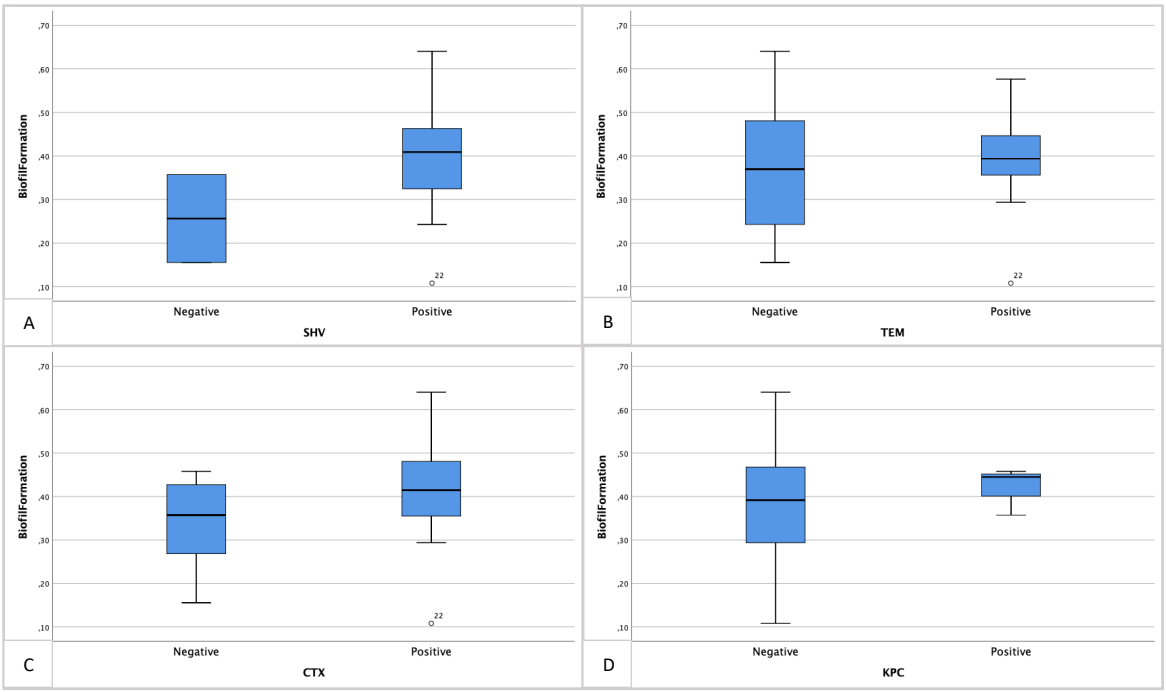

Figure 4.3- Boxplots that display biofilm formation data for the presence of different resistance genes. A) SHV; B) TEM; C) CTX; D) KPC. The black bars in the boxes represent the median values.

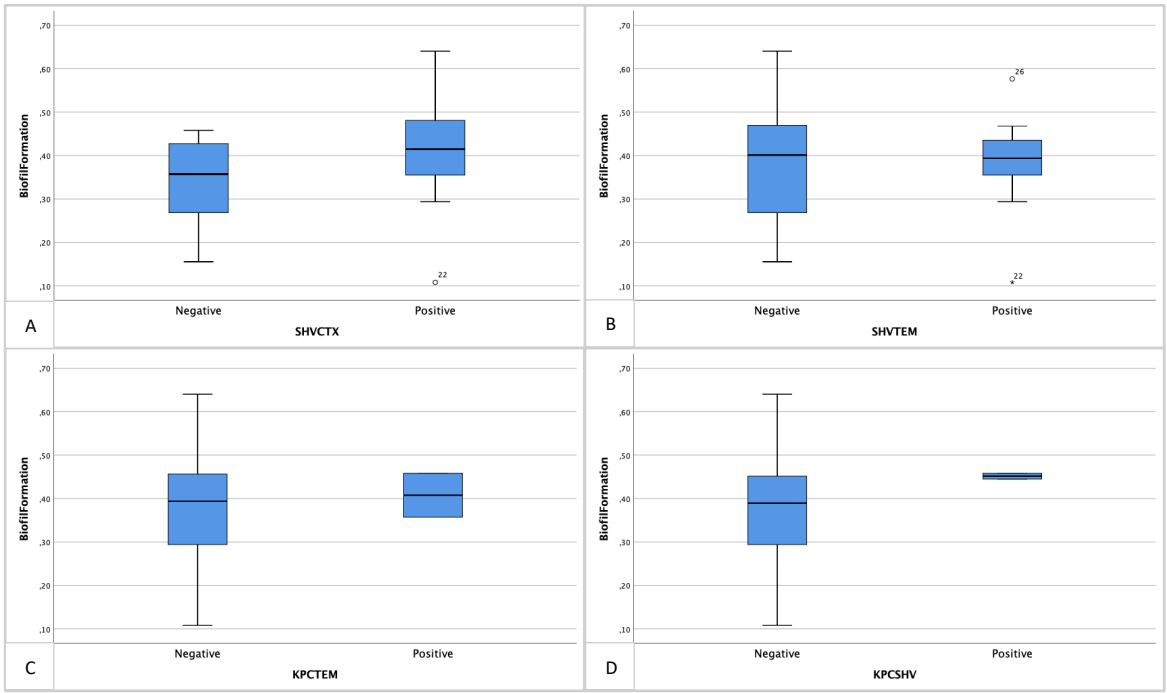

Figure 4.4- Boxplots that display biofilm formation data for the presence of different double combinations of resistance genes. A) SHVCTX; B) SHVTEM; C) KPCTEM; D) KPCSHV. The black bars in the boxes represent the median values.

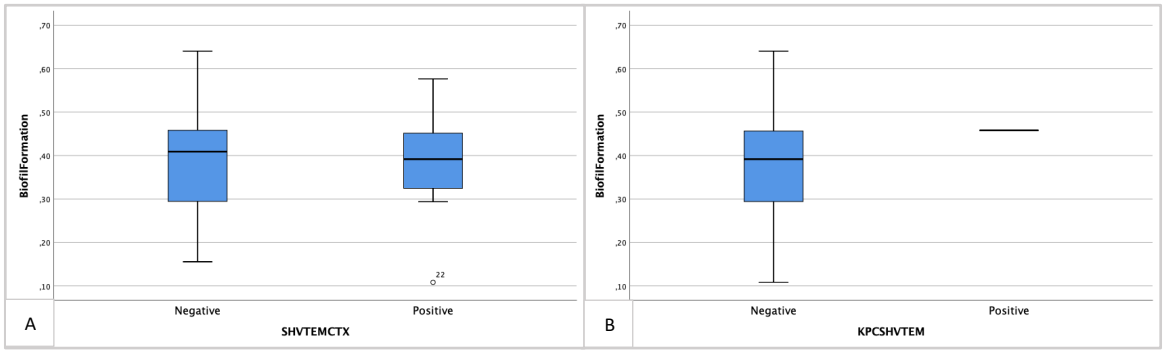

Figure 4.5- Boxplots that display biofilm formation data for the presence of different triple combinations of resistance genes. A) SHVTEMCTX; B) KPCSHVTEM. The black bars in the boxes represent the median values.

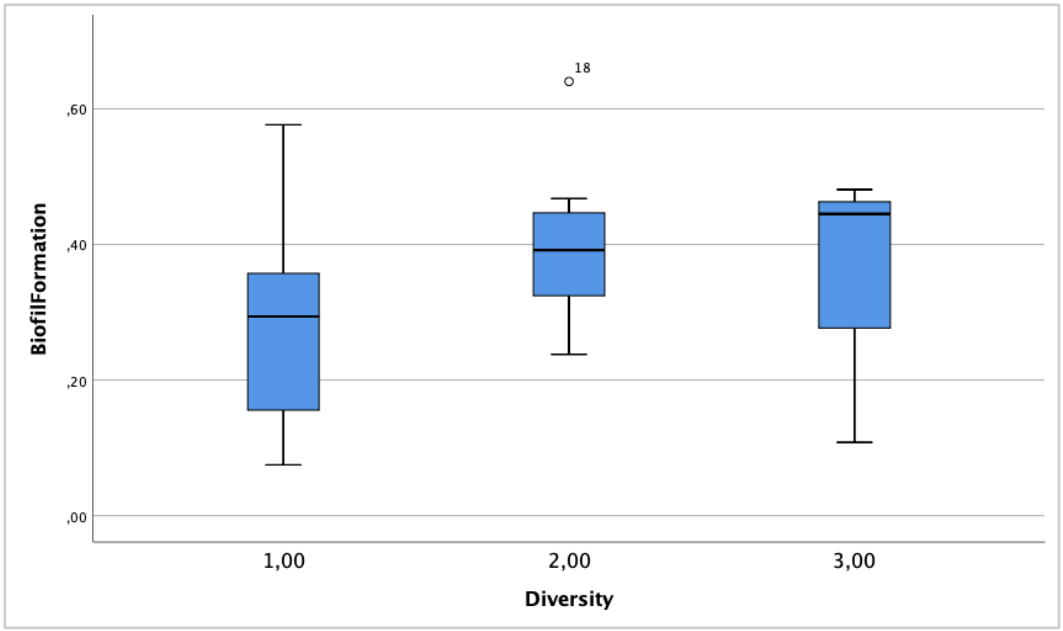

Figure 4.6- Boxplots that display biofilm formation data for the diversity of morphotypes. The black bars in the boxes represent the median values.

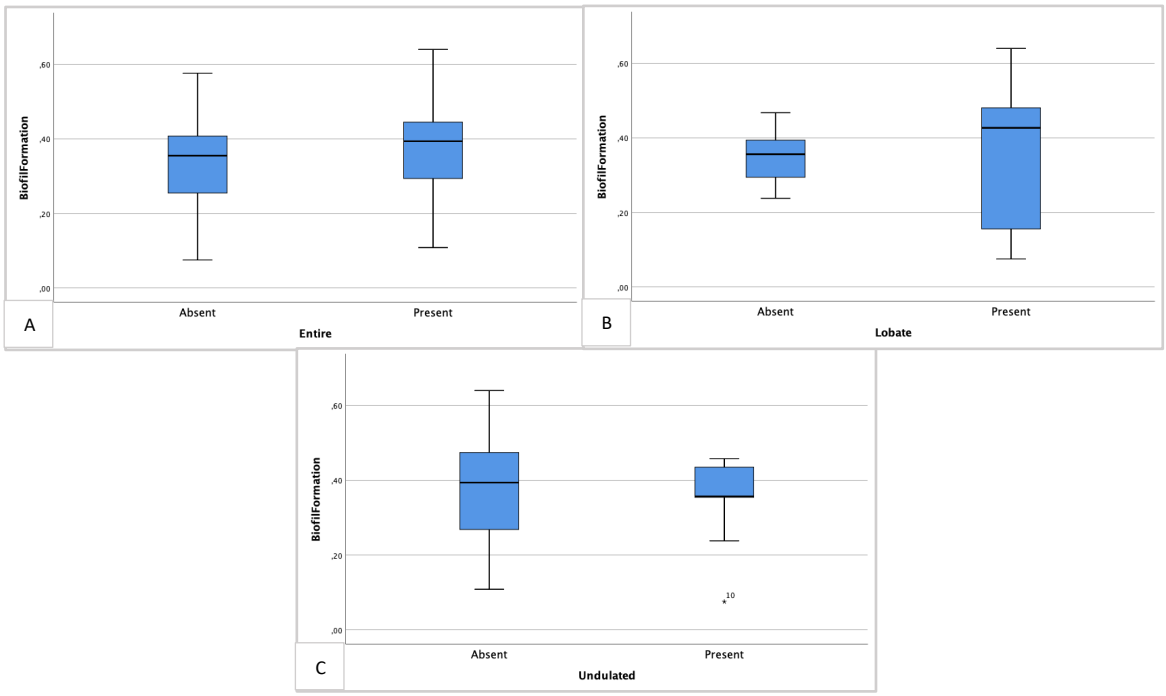

Figure 4.7- Boxplots that display biofilm formation data for the presence or absence of different types of margin in the colonies. A) Entire; B) Lobate; C) Undulated. The black bars in the boxes represent the median values.

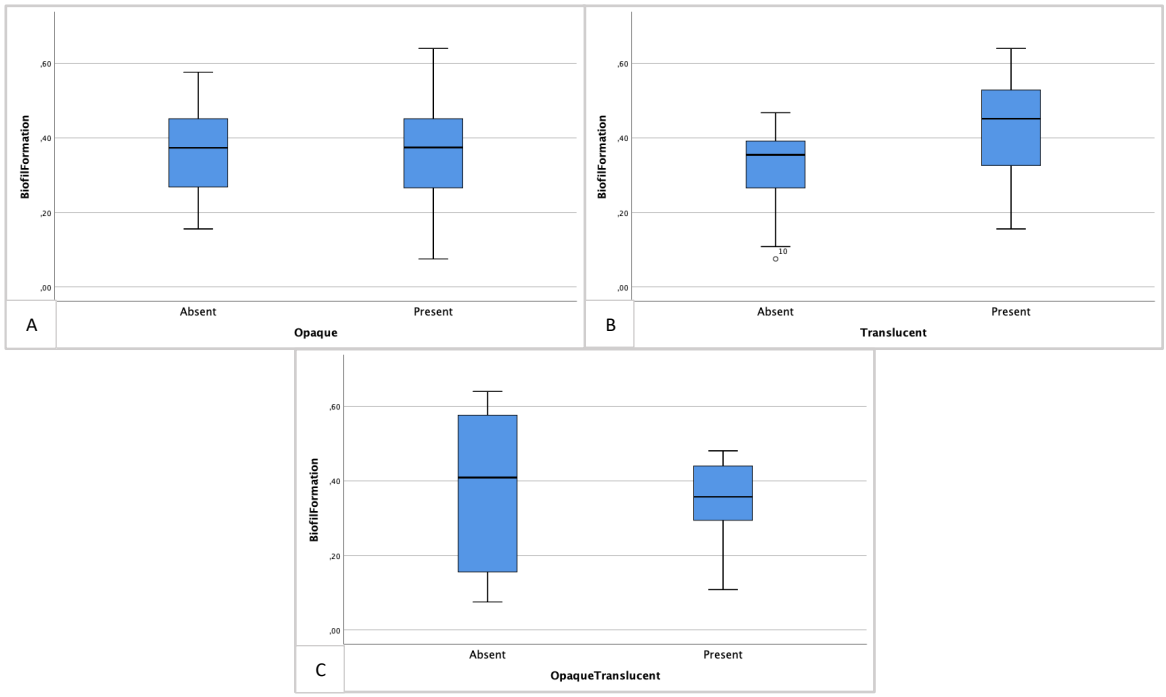

Figure 4.8- Boxplots that display biofilm formation data for the presence or absence of different types of opacity in the colonies. A) Opaque; B) Translucent; C) Opaque and Translucent. The black bars in the boxes represent the median values.